CALCIUM IONS AND VISUAL EXCITATION

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Biochimica et Biophysica Acta, 552 (1979) 379—389

BBA 78333

THE ISOLATION OF STABLE CATTLE ROD OUTER SEGMENTS WITH AN INTACT PLASMA MEMBRANE

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(Received June 12th, 1978)
(Revised manuscript received November 28th, 1978)

Key words Rhodopsin, Photolysis, Vision, (Intact rod)

Summary

A procedure is described to purify and stabilize cattle rod outer segments with an intact plasma membrane. Three criteria are applied to assess the integrity of the latter.

Upon photolysis in these rod outer segments: (1) exogenous ATP cannot phosphorylate rhodopsin located in the disk membrane. (2) Endogenous cofactors (NADPH, NADPH-regenerating system) are still available in the rod cytosol and consequently retinol is the final photoproduct of photolysis of rhodopsin. (3) The rod cytosol can maintain a pH different from that of the medium, since the later stages of rhodopsin photolysis are independent of the medium pH.

The stability and homogeneity of the preparation appear to be much better than those of freshly isolated frog rod outer segments, which have been used most frequently so far for experiments on the physiology of rod outer segments. In addition, these cattle rod outer segments remain intact during various manipulations and therefore considerably extend the experimental possibilities when intact rod outer segments are required.

Introduction

Vertebrate rod outer segments (rods) are connected by a narrow cilium to the rest of the photoreceptor cell. Upon mechanical agitation the outer segment easily breaks off and can be conveniently purified by means of density gradient centrifugation. It has generally been assumed that the enveloping plasma membrane reseals during this procedure. This assumption is crucial for

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experiments on the physiology of isolated rods and hence it is surprising, that so few attempts have been made to check this assumption. The criterion presently available to assay the intactness of the plasma membrane, the dye staining test [1], has only been used in a few recent studies [2—4] and exclusively for frog rods. The authors agree that even freshly isolated frog rods contain 30—70% leaky rods and the number of leaky rods increases upon manipulations (e.g. centrifugation and resuspension). Consequently purification of frog rods with an intact plasma membrane seems impossible when a 'physiological' isolation medium is used.

Cattle rods, isolated in Tris buffer and purified by sucrose density gradient centrifugation, appear completely leaky to small solutes like ATP [5]. Furthermore, the resulting preparation deteriorates fast (and may already be deteriorated to a considerable extent during isolation) upon manipulation and ageing with respect to two important parameters of calcium metabolism of rods, calcium storage capacity and calcium translocation capacity [5].

We have now devised isolation conditions which stabilize the rod structure and allow purification of rods, which appear stable and not deficient with respect to calcium metabolism. In addition, the integrity of the enveloping plasma membrane can be maintained. To assess the latter we have applied three criteria, based on the permeability properties of the plasma membrane to small solutes.

Materials and Methods

Isolation procedure for cattle rods with an intact plasma membrane. All procedures are carried out in darkness or in dim red light. Plastic labware is used throughout the procedures. Cattle eyes are collected at the local slaughterhouse as fresh as possible (frozen retinas are not suitable). The retinas are carefully dissected and collected in ice-cold isolation medium (0.5 ml/retina), containing 600 mM sucrose, 5% Ficoll 400, 10 mM D-glucose, 10 mM ascorbic acid, 1 mM $CaCl_2$, 20 mM Tris-HCl (pH 7.4). $CaCl_2$ acts as a stabilizing agent and ascorbate as an anti-oxidant. Before addition of the other components the sucrose/Ficoll solution is passed over a mixed-bed ion-exchange column. The outer segments are shaken off on a vortex mixer (30 s at maximal speed) and filtered through a teflon screen (125 mesh). The filtrate is applied on top of a gradient (7 ml/gradient) by means of a syringe fitted with plastic tubing (inner diameter 3 mm). The continuous gradient is made up by mixing equal volumes (13 ml/gradient) of isolation medium and 20% (w/w) sucrose, 16% (w/w) Ficoll 400.

The gradients are centrifuged for 1 h at 24 000 rev./min (40 000—100 000 × *g*) in an IEC B-60 ultracentrifuge (5—10°C). The rod-containing band is collected in a syringe fitted with plastic tubing, is diluted with two volumes of 600 mM sucrose, 20 mM Tris-HCl (pH 7.4) and centrifuged (20 min, 3000 × *g*, 5—10°C). An occasionally occurring upper band, containing rhodopsin, is discarded. The pellet is carefully resuspended in 600 mM sucrose, 5% Ficoll 400, 20 mM Tris-HCl (pH 7.4), to a final concentration of about 100 μM rhodopsin and stored at 4°C.

Spectral recordings. Spectral recordings of suspensions are performed on a

Rapid T3 spectrophotometer (Howaldtswerke-Deutsche Werft, Kiel, F.R G.) or a Pye Unicam SP1750 UV spectrophotometer.

Spectral ratios are determined in 1% Ammonyx LO Absorbance at 278 nm due to the presence of ascorbate can be reduced by the addition of hydrochloric acid (final concentration 0 3 M) after the 500 nm absorbance has been measured Rhodopsin determinations are performed as described before [6].

The time course of photoproduct formation (see Fig 4) is analyzed at three wavelengths 330, 380 and 455 nm. In order to calculate the concentrations of the individual photoproducts retinol (λ_{max} = 330 nm), metarhodopsin II + all-*trans* retinal (λ_{max} = 380 nm) and metarhodopsin III (λ_{max} = 455 nm) with respect to the amount of rhodopsin photolyzed (separately determined) from the absorbance changes at these three wavelengths, the following procedure is used. The molar extinction coefficients of rhodopsin, metarhodopsin II + all-*trans* retinal, and metarhodopsin III are taken to be the same [7] and a value of 40 600 is used. The molar extinction coefficient of all-*trans* retinol (52 600 in ethanol) was found to be depressed by 25% when added to an aqueous suspension of rod membranes, concomitant with a shift of λ_{max} from 325 to 330 nm. To obtain changes of metarhodopsin II + all-*trans* retinal the absorbance changes at 380 nm are corrected for changes at 380 nm due to metarhodopsin III (25% with respect to 455 nm) To obtain changes of retinol the absorbance changes at 330 nm are corrected for changes of metarhodopsin II + all-*trans* retinal (38% with respect to 380 nm) and for changes of metarhodopsin III (20% with respect to 455 nm). The correction coefficients used (others are small and have been neglected) are estimated from nomograms of all-*trans* retinal and protonated retinylidene-phosphatidylethanolamine (van Breugel, P., unpublished results). These are similar to those used by Baumann [8], with the exception of the contribution of metarhodopsin III at 330 nm. These corrections may not be completely appropriate, but the final result appears to be subject to small errors only. This is illustrated by the fact that with water-washed rod membranes, in the absence of retinol formation, the absorbance changes observed at 330 nm agree within 10% with those calculated from the decay of metarhodopsin II and the formation of metarhodopsin III. Furthermore, during the first 15 min after photolysis the sum of the photoproducts amounts to 98—104% with respect to the rhodopsin photolyzed and then decays slowly to 86 5% after 60 min. This is most probably due to degradation of the retinol formed. For obvious reasons, the calculation is only fairly accurate if the scattering of the suspension (monitored at 650 nm) does not change during the measurement.

Other procedures. Rods with a leaky plasma membrane are prepared as described before [5,6], involving mild homogenization in a Tris-HCl buffer (0.16 M, pH 7.4), followed by sucrose density gradient centrifugation and washing with the same Tris-HCl buffer. A modified procedure to prepare stable leaky rods is discussed in the Results section.

Phosphorylation of rhodopsin with [γ-^{32}P]ATP is determined as described before [5].

Dodecyl sulfate disc gel electrophoresis is carried out according to Weber and Osborn [9] with 8% gels. Proteins are stained with Coomassie blue R250.

Results

General characterization of the preparation

The best preparations are invariably prepared from very fresh eyes. Under these conditions the density gradient shows, about half-way, one major band, which is used. An occasionally occurring upper band, often containing straight rods without shrunken appearance, and some material left on top of the gradient are carefully removed by suction prior to collection of the major band. The gradient does not contain further particulate material and ends in a small, tight pellet, reddish brown at the top and black at the bottom.

Examination of our rod outer segment preparations by phase contrast microscopy shows that the large majority of the particles can be identified as rods or rod fragments, but does not allow quantitative conclusions about the purity of the preparations or about the intactness of the plasma membrane. Only very few rods carry a part of the inner segment. By far the most rods give the impression to be shrunken and to be somewhat bended, undoubtedly by the hypertonic conditions. The amount of smaller rod fragments does not appear to affect the percent of leaky rods (see further).

The purity of the intact rod preparation is characterized by the spectral ratio A_{278}/A_{500} and by SDS gel electrophoresis. After a single lysis step to remove soluble cytosol proteins (and ascorbate, which gives considerable absorbance at

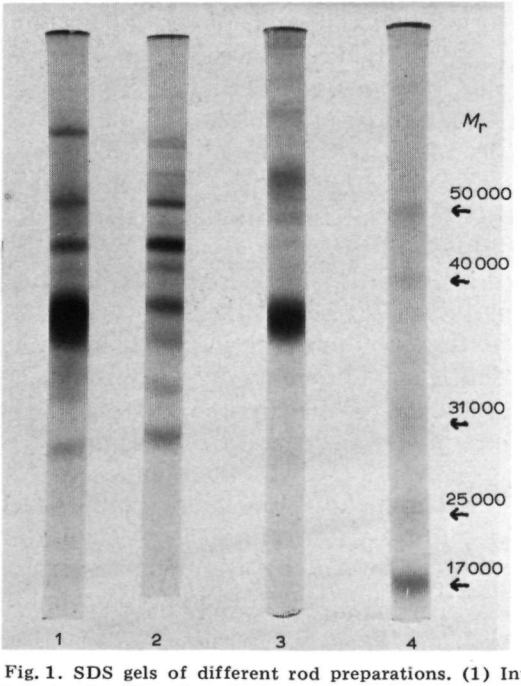

Fig. 1. SDS gels of different rod preparations. (1) Intact rods; (2) supernatant, obtained after lysis of intact rods; (3) pellet, obtained after lysis of intact rods, and (4) calibration gel. Intact rods, resuspended in the standard medium, are lysed by addition of 15 volumes of water. After addition of a sodium phosphate buffer (pH 7.0) to a final concentration of 40 mM the membranes are centrifuged for 16 h at 140 000 × *g*. The supernatant is dialyzed against water and subsequently concentrated. The concentrated supernatant does not contain a detectable amount of rhodopsin.

278 nm), the A_{278}/A_{500} ratio of these preparations lies routinely between 1.9 and 2.3, indicating a reasonable purity [6,10,11]. In intact rods 20—30% more protein may be present, as judged by the absorbance at 278 nm (after acid treatment), but this may still be partly due to ascorbate.

SDS gels (Fig. 1) of intact rods consistently show, as compared to lysed rods, the same number of additional bands, which appear in the 100 000 × *g* supernatant after lysis. This might indicate that they are soluble cytosol proteins. Leaky rods also contain these bands, though to a variable degree. The plasma membrane in these preparations apparently retains these large molecules in contrast to smaller molecules like ATP and NADPH (see next sections).

Criteria for the intactness of the plasma membrane

The dye staining test [1] unfortunately does not work with cattle rods. Isolated cattle rods do not stain with didansylcysteine irrespective of the state of the plasma membrane. A similar observation has been made by P. Hochstrate and H. Rüppel (personal communication). Therefore we have used three other criteria to assess the integrity of the plasma membrane: entry of exogenous ATP, retention of endogenous NADPH, and retention of protons. They are all based on the degree to which the plasma membrane forms a permeability barrier to small hydrophilic solutes.

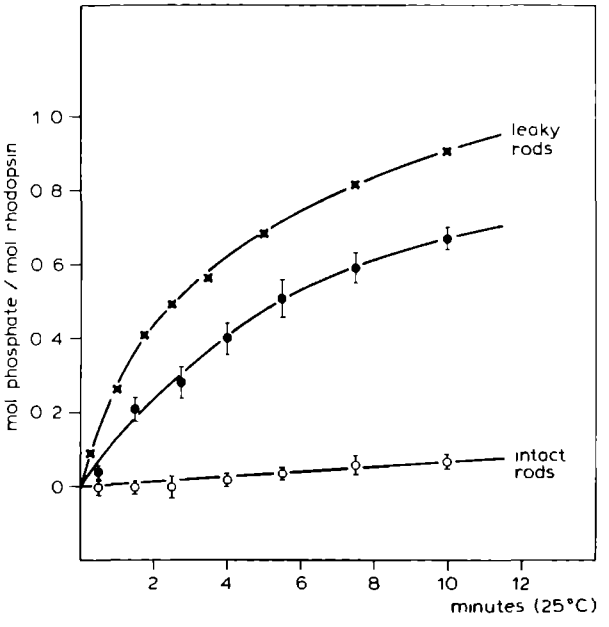

Fig. 2. Light-dependent phosphorylation of isolated cattle rods. In addition to the media, 2 mM $MgCl_2$, 1 mM Tris/ATP and [γ-^{32}P]ATP. Illumination is started 30 s before addition of ATP and sustained until complete bleaching. All data are corrected for phosphate incorporation in the dark (in leaky rods maximally 0.1 mol phosphate/mol rhodopsin in intact rods virtually absent). X, leaky rods, resuspended in 100 mM KCl, 20 mM Tris-HCl (pH 7.4), data from [5], ○, intact rods in 600 mM sucrose, 5%(w/v) Ficoll 400, 20 mM Tris-HCl (pH 7.4), ●, intact rods, resuspended in 200 mM KCl, 20 mM Tris-HCl (pH 7.4). The latter two represent averages ± S.F. of 3—6 experiments with different preparations. Temperature 25° C.

Entry of exogenous ATP

Upon photolysis rhodopsin can be phosphorylated with ATP by an intrinsic kinase, resulting in a phosphate ester, which is stable in vitro [12,13].

Hence, a suitable reaction to test the permeability to ATP is the phosphorylation of photolyzed rhodopsin, located in the disk membrane, with exogenous [γ-^{32}P]ATP. Fig. 2 shows that our intact rod preparations cannot be phosphorylated by exogenous ATP. Resuspension of these rods in an electrolyte medium makes the phosphorylation site accessible to [γ-^{32}P]ATP. The phosphorylation seems somewhat slower than in the leaky rod preparations used before [5]. Phosphorylation can also be observed when leaky rods are resuspended in the sucrose/Ficoll medium.

Retention of endogenous NADPH

If endogenous cofactors like ATP cannot enter the rod, conversely endogenous cofactors like NADPH should still be present in the rod cytosol. In the vertebrate retina, free and opsin-bound all-*trans* retinal, formed upon photolysis of rhodopsin, are reduced to all-*trans* retinol [8,14,15] by an intrinsic retinol dehydrogenase with NADPH as cofactor [16].

Fig. 3 demonstrates that after 80% bleaching of rhodopsin retinol (λ_{max} = 330 nm) is the final photoproduct in the intact rod preparation, while in leaky rods a mixture of all-*trans* retinal (λ_{max} = 380 nm) and metarhodopsin III (λ_{max} = 455 nm) has formed. Fig. 4 shows the time course of photoproduct formation after a 50% bleach. The difference between intact and leaky rods is

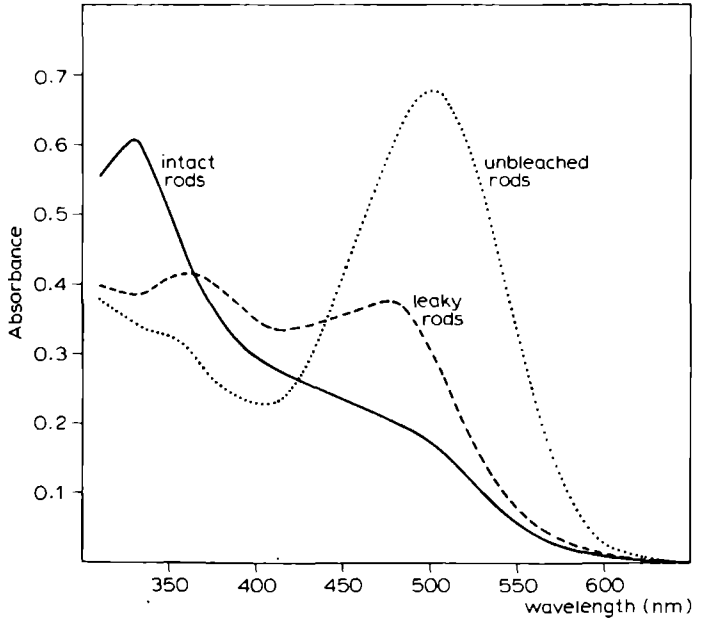

Fig. 3. Final photoproducts in intact and leaky rods. Suspension spectra are shown, which are normalized so that the rhodopsin spectra coincide. The absorbance at 650 nm is set to zero, · · · · · ·, unbleached rods; ———, intact rods following 80% photolysis; - - - - - -, leaky rods following 80% photolysis. Medium: 600 mM sucrose, 5% (w/v) Ficoll 400, 20 mM Tris-HCl (pH 7.4). Temperature: 25° C.

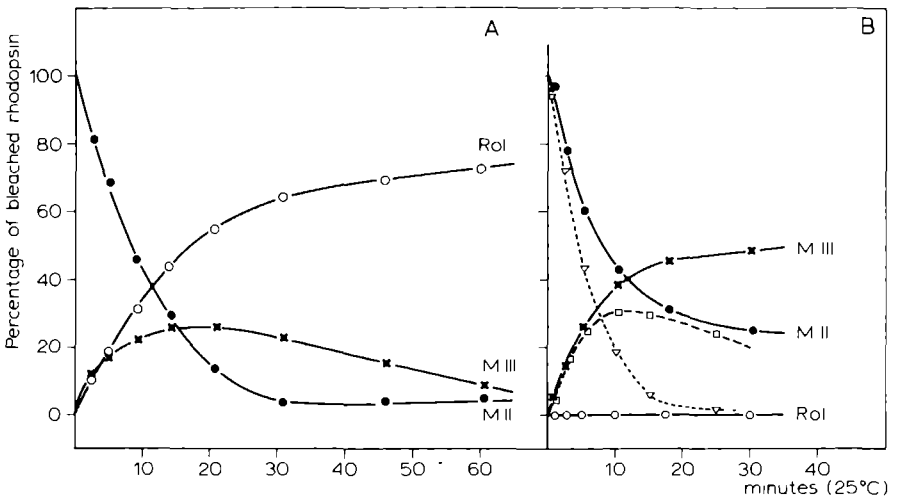

Fig. 4. Time course of photoproduct formation following 50% photolysis, in intact (A) and leaky rods (B). Retinol ( ), metarhodopsin II + retinal (●); metarhodopsin III (X), leaky rods metarhodopsin II + free retinal, with a 10-fold molar excess of added NADPH (○), leaky rods· metarhodopsin III with a 10-fold molar excess of added NADPH (□). Medium 600 mM sucorse, 5% (w/v) Ficoll 400, 20 mM Tris-HCl (pH 7.4). Temperature 25° C.

obvious. At lower bleaches relatively less metarhodopsin III is formed (not shown), similar to what has been described for the frog retina [17].

Fig. 4b shows that in leaky rods excess exogenous NADPH gives rise to a faster disappearance of 380 nm and 455 nm absorption (because of the overlapping absorption spectra of NADPH and retinol, retinol formation cannot be analyzed). This suggests, that in intact rods regeneration of NADPH is the rate-limiting step. In agreement with this, NADPH cannot be detected spectrophotometrically in the preparation, although nearly complete reduction of all the chromophore can be achieved. Hence, a considerable activity of a NADPH-regenerating system from glucose and ATP, which is specific for $NADP^+$ over NAD^+ can be demonstrated in our intact rods.

Retention of protons

Our interest in ion fluxes in rods has inspired us to look for a permeability test involving small ions. The pH dependence of the metarhodopsin I (λ_{max} = 480 nm) ⇌ metarhodopsin II (λ_{max} = 380 nm) equilibrium [7] can be used to establish whether the rod plasma membrane can act as a proton barrier and maintain a proton gradient.

Fig. 5 shows that in leaky rods exposure of metarhodopsin II to an external pH of 9 at 0°C results in a spectral shift to longer wavelengths, indicating a shift of metarhodopsin II to a pigment, isochromic with metarhodopsin I. Reduction of the temperature to 0°C without a change of external pH leaves metarhodopsin II unaffected. In intact rods metarhodopsin II persists when the external pH is adjusted to 9 at 0°C. From the subsequent disappearance of metarhodopsin II a time constant for the proton leakage through the rod plasma membrane in the order of minutes can be estimated. Upon addition of

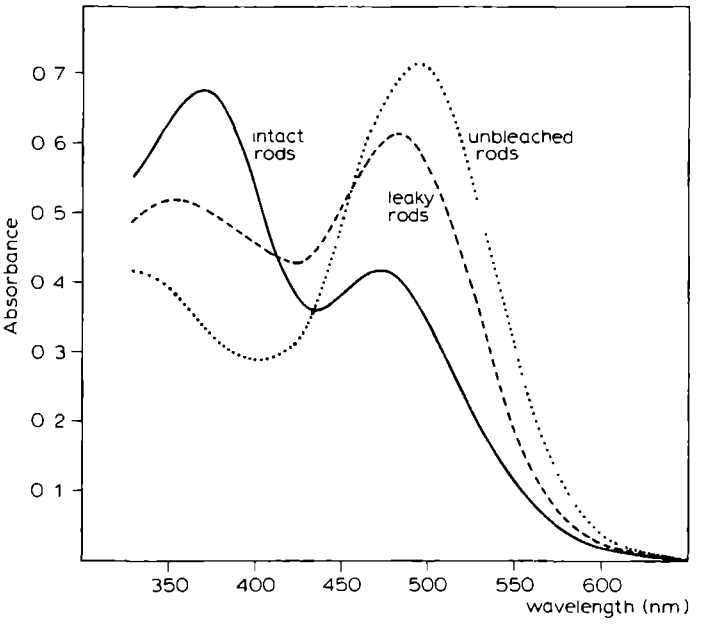

Fig. 5. pH dependence of the later stages of photolysis of rhodopsin in intact and leaky rods. A concentrated rod suspension (100 µl) in 600 mM sucrose, 5% (w/v) Ficoll 400, 20 mM Tris-HCl (pH 7.4), is bleached during 5 s (approx. 80% photolysis) at 25° C. Immediately 900 µl icecold 600 mM sucrose 5% (w/v) Ficoll 400, 50 mM Tris-HCl (pH 9.0) is added and a spectrum is recorded. The spectra of both preparations are normalized so that the rhodopsin spectra coincide. Absorbance at 650 nm is set to zero. ·····, unbleached rods; ———, intact rods following photolysis, - - - - -, leaky rods following photolysis.

NH_4Cl (10 mM) to the external medium the existing proton gradient across the rod plasma membrane is equilibrated in seconds, resulting in a similar spectral shift to longer wavelengths as observed with leaky rods.

Stability of intact and leaky cattle rod outer segments

Intact cattle rods stored as a concentrated suspension in the sucrose/Ficoll medium remain intact for several days at 4° C. The structure still seems unaffected when viewed by phase contrast microscopy and electronmicroscopy and no phosphorylation by exogenous ATP can be observed. Formation of retinol is slowed down (about 50% after 24 h at 4° C), but still occurs to about the same extent after two days at 4° C. This indicates that the NADPH level has been lowered, but that metabolites required for NADPH regeneration are still sufficiently available. Intact rods remain inaccessible to exogenous NADPH. This can be demonstrated by following the time course of metarhodopsin III formation and decay with and without exogenous NADPH (similar as carried out in Fig. 4b).

The calcium storage and translocation capacities are also well preserved. The preparation can be subjected to various manipulations such as complete photolysis of rhodopsin, centrifugation and resuspension, sampling, osmotic manipulation, partial substitution of sucrose by electrolytes, without affecting the integrity of the plasma membrane, although the stability may be reduced (Schnetkamp, P.P.M., unpublished data).

When leaky rods are resuspended (after gradient centrifugation) in the same sucrose/Ficoll medium as used for the intact rods, the resulting preparation remains leaky according to the three criteria, but parallels intact rods as far as stability of structure and as far as calcium storage and translocation capacities are concerned.

Discussion

An essential prerequisite for experiments on the physiology of isolated rod outer segments (rods) seems to be the availability of a pure, stable and homogeneous preparation of rods with either an intact or leaky plasma membrane. Freshly isolated frog rods have been used in most studies up to now. This preparation contains 30—70% leaky rods as indicated by the dye staining test [1—4]. To our knowledge no information is available on the purity of these preparations. Furthermore three important properties, which have been analyzed in these preparations, have yielded rather conflicting results. The calcium content of isolated frog rods may vary over almost two orders of magnitude [4,18]. Reports of light-induced calcium release [4,18,19] have not always been substantiated. Osmotic experiments [20,21] have indicated a selective permeability of the plasma membrane to NaCl with respect to KCl, but others [2,22] have failed to reproduce this finding.

Application of the three criteria for the intactness of the plasma membrane used in this study shows that freshly isolated frog rods exhibit retinol formation, which is lost after a single washing step [23,24]. In our hands cattle rods, freshly isolated in a Ringer solution, show little retinol formation upon photolysis of rhodopsin as compared to cattle rods, freshly isolated in the sucrose/Ficoll medium. With microspectrophotometry Kaplan and Liebman [3] observed that only frog rods, which do not stain with didansyl cysteine, form retinol. Both criteria therefore seem to give comparable results. According to Bownds and coworkers [24,25] freshly isolated frog rods (even without washing) can be phosphorylated by exogenous ATP. On the other hand, Robinson et al. [26] and Carretta and Cavaggioni [27] find that endogenous ATP (or high-energy phosphate, Ref. 28) remains associated with frog rods, also after washing and even after gradient centrifugation. Bridges [23] reports that both fresh and washed frog rods are permeable to protons and NADPH. It is clear from these findings that freshly isolated frog rods do not give consistent results.

The present study describes a procedure to stabilize and purify cattle rods with an intact plasma membrane. The choice of hypertonic sucrose as the major osmotic component in the medium stems from practical and empirical considerations. An analogy to the isolation of functionally intact mitochondria and chloroplasts is clear. Mitochondria are always isolated in sucrose media and in the case of chloroplasts sucrose appears superior to electrolytes [29,30]. The deleterious effect of electrolytes on rods is illustrated in Fig. 2, where it is shown that mere resuspension of intact rods in a KCl medium makes them leaky. Addition of Ficoll 400 has a further stabilizing effect, as with mitochondria and chloroplasts, and it enables the use of a density gradient of constant osmotic strength. Furthermore a procedure is provided to prepare stable leaky

rods, which seem only deficient as compared to intact rods as far as the integrity of the plasma membrane is concerned Both preparations appear of good purity as indicated by spectral ratios and SDS gels.

The three intactness criteria indicate that the plasma membrane in our intact rods has resealed and constitutes a permeability barrier to ATP, NADPH and protons Both from the phosphate incorporation (Fig 2) and from the remaining all *trans* retinal (Fig 4) we conclude that less than 10% of the rods are leaky after gradient centrifugation and washing This value seems not considerably increased by ageing (up to several days at 4°C) and manipulations (complete photolysis of rhodopsin, centrifugation and resuspension, sampling, partial medium substitution by electrolytes) Therefore the homogeneity and stability of these rods are much better than reported for freshly isolated frog rods [2—4]

Isolation of cattle rods according to the procedure described in this study thus yields a stable rod preparation with intact plasma membrane. The rod cytosol contains sufficient metabolites and the enzymes for the generation of the NADPH required to reduce all the chromophore, released upon photolysis of rhodopsin. The resulting time course of the formation of the slow photoproducts resembles those observed in the retina [8,14,15,17] This preparation and the stable rods with a leaky plasma membrane have already been useful and shown to be superior to conventional preparations in the study of the 'dark calcium metabolism' of rods (Schnetkamp, P.P M , unpublished data) They also show reproducible and fast calcium shifts in response to light, which are lost in other preparations [31].

Acknowledgements

Our thanks are due to Dr. W J. de Grip, Dr P J G.M. van Breugel, Prof. Dr S L Bonting and Mr P.A A Jansen for critical discussions during the course of the work and during the preparation of the manuscript. This investigation is supported in part by the Netherlands Organization for the Advancement of Basic Research (Z.W.O.), through the Foundation for Chemical Research in the Netherlands (S.O N.)

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Biochimica et Biophysica Acta, 552 (1979) 390—403

BBA 78334

LIGHT-INDUCED CALCIUM RELEASE IN ISOLATED INTACT CATTLE ROD OUTER SEGMENTS UPON PHOTOEXCITATION OF RHODOPSIN

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(Received July 10th, 1978)

Key words Rhodopsin, Ca^{2+} release, Vision, Photoexcitation, (Bovine rod outer segment)

Summary

By applying flash-spectrophotometry with the calcium-indicating dye arsenazo III rapid light-triggered calcium release in various cattle rod outer segment preparations was studied. It is shown that light-induced calcium signals can be unambiguously discriminated from underlying absorption changes due to photolysis of rhodopsin and apparent absorption changes resulting from light-scattering transients.

The following results have been obtained:

1. Calcium-induced arsenazo III responses can be quantitatively and kinetically resolved within the time domain of the visual transduction process.

2. Photoexcitation of rhodopsin results in calcium release from intradiscal binding sites.

3. Calcium released does not appear in the cytoplasmic space unless the disc membrane is made permeable to calcium ions by an ionophore.

4. The shortest observed half-rise time of calcium release (300 ms) is possibly limited by the ionophore.

5. The stoichiometric ratio of calcium released/rhodopsin bleached is 0.5 at a free calcium concentration of 2 μM. The amount of calcium released is proportional to the percentage of rhodopsin bleaching (from 1—10%).

6. Upon disruption of the disc stack by lysis of intact rod outer segments the light-induced calcium release is greatly altered.

The results are discussed in relation to previous reports on a light-induced calcium release from retinal discs and in terms of the proposed role of calcium as an intracellular transmitter in vertebrate photoreceptors.

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Introduction

In 1971, Yoshikami and Hagins proposed that calcium is the primary transmitter between the absorption of a quantum of light by rhodopsin and the electrical response of the outer membrane of vertebrate rods [1] The hypothesis was proposed because extracellular recordings of the voltage drop along the length of the outer segment suggested that raising the intracellular calcium concentration had the same inhibitory effect on the sodium current across the cell envelope as photoexciting the rhodopsin [2,3]. Intracellular recordings of the membrane potential of vertebrate photoreceptor cells in the dark and during illumination have provided additional evidence in favour of this notion, although they do not prove the hypothesis [4,5,6]

Considerable work has also been expended in attempting to detect calcium release and accumulation from a variety of photoreceptor preparations using atomic absorption spectroscopy [7—10], ^{45}Ca techniques [11—19], calcium-sensitive electrodes [20] and flash-spectrophotometry with the metallochromic dye arsenazo III [21] These various preparations, including lysed and sonicated material, have yielded conflicting results. Some authors did not detect any light effect on the redistribution of intracellular calcium [10,11,14,37] When a light-induced calcium release was measured the reported stoichiometric ratios of calcium released/rhodopsin bleached were highly divergent, a fact which might be attributed to the large differences between the preparations used [7,13,15,16,19,20,21] It is noteworthy that only after previous fragmentation of the disc stack a light effect could be observed [7,13,15,19—21], but not in rod outer segments with an intact stack of discs [7,11,37], in which the outer membrane has been shown to be leaky [10,22—26]

Although it seems established that light has some effect on the redistribution of calcium in retinal rods, unequivocal evidence that calcium is released with the stoichiometry required for the transmission and especially within the time domain of the visual transduction process is still lacking

We have recently reported a rapid calcium release from passively loaded retinal disc vesicles detected by flash-spectrophotometry with the calcium-indicating dye arsenazo III and with the above mentioned time resolution [21]. The stoichiometric ratio of 1 calcium released/30 rhodopsin molecules bleached measured in this study was far too low to be correlated with any functional role of calcium in visual transduction

Using the same technique in this paper we have further examined the effect of light on the redistribution of intracellular calcium in rod outer segments well characterized with respect to the integrity of the plasma membrane and the disc stacking In single flash experiments calcium-induced arsenazo III responses have been resolved with bleaching percentages down to 0 5% of the rhodopsin

Materials and Methods

Preparations Three types of isolated cattle rod outer segment preparations were used. They were stabilized and purified according to Schnetkamp et al. [26] and stored as a concentrated suspension (100 μM rhodopsin). The preparations differ in the permeability of the plasma membrane to small solutes

and in the integrity of the disc stacking and are referred to as 'intact', 'leaky' and 'lysed' rod outer segments Intact rods have a plasma membrane functioning as a permeability barrier to ATP, NADPH and protons, whereas in leaky rods no permeability barrier to these solutes appears to be present [26] Both preparations have a morphologically intact stack of discs [25,26]. Lysed rods are prepared by addition of 10 volumes of distilled water to an intact rod outer segment suspension and subsequent centrifugation and resuspension The majority of the discs remains closed and retains calcium upon lysis, although the stack of discs is disrupted [27] The endogenous calcium content of leaky and intact rod outer segments as determined by atomic absorption spectroscopy (1 mol calcium/mol rhodopsin and 2—3 mol calcium/mol rhodopsin [25,27]) could be confirmed by spectrophotometric determinations with arsenazo III. Intact rod outer segments, preloaded with ^{45}Ca, retain about 60—75% of their endogenous calcium content upon lysis by a hypoosmotic shock [27] All preparations were resuspended and diluted in a standard medium, containing sucrose, 600 mM, Ficoll-400, 5% and Tris-HCl buffer, 20 mM at pH 7 4

Experimental procedures For the measurements rod outer segments were diluted to a final rhodopsin concentration of 5 μM Rhodopsin concentrations were determined according to de Grip et al [28]. The number of rhodopsin molecules bleached/flash was determined in the flash-photometer in the presence of 30 μM arsenazo III from the appearance of metarhodopsin II at 382 nm, using the same molar extinction coefficient ϵ = 40 000 mol/cm^2 as for rhodopsin at 498 nm [29] Ionophore A23187 was a gift of Eli Lily Co. (Giessen) and was used without further purification in an ethanolic solution. The final concentration of A23187 in the cuvette was 10 μM if not otherwise indicated. The ethanol concentration introduced into the suspension by the ionophore stock solution was $\leqslant$1% v/v.

The incubation time was about 2 min. After this time all of the calcium stored within discs has been equilibrated across the disc and plasma membranes via the ionophore. The calcium efflux from rod outer segments after addition of ionophore was followed spectrophotometrically The temperature was always 20°C

Generally a 2 ml cuvette with 10 mm pathlength was used For the experiments with different bleaching levels a 0 5 ml cuvette with a different geometry was used (10 mm pathlength of the measuring light but only 2 mm pathlength of the exciting light). The absorbance of the suspension in the direction of the measuring light beam was A = 1.0—1.3 at 655 nm. In the 0.5 ml cuvette the absorbance in the direction of the excitation flash was 0.2 at 530 nm (excitation wavelength). This low absorbance allows for an uniform bleaching throughout the cuvette by the laser flash. If the cuvette were 10 mm wide an exciting flash could give rise to a bleaching gradient along the light path of the flash and recordings of the flash-induced absorption changes could represent integration over a broad bleaching range. The laser beam was expanded to such an extent that uniform bleaching of the whole cross-section of the cuvette was achieved.

In all recordings shown, the sample was exposed only to the one very flash without previous illumination .

Calibration of arsenazo III Arsenazo III was purchased from Sigma (Munich, grade I, M_r = 776 4) and used without further purification The calcium contamination was found to be less than 5% The arsenazo III concentration in a cuvette was always 30 μM. To calibrate the light-induced absorption changes into μM released calcium, a certain amount of calcium was added to a rod outer segment suspension and the change in absorption measured The calibration procedure was performed under similar conditions to those used for the flash-spectrophotometric experiments, i e in the presence of 30 μM arsenazo III, 10 μM A23187 and rod outer segments which contained 5 μM rhodopsin A change in the relative transmission $\Delta I/I$ = 3 10^{-2} was observed upon addition of 1 μM calcium to a total (bound + free) calcium concentration of 15 μM Up to a total calcium concentration of 20 μM, the absorbance of arsenazo is linearly related to the total amount of calcium present. Thus the differential sensitivity $\Delta A/\Delta$Ca or arsenazo III is almost constant within the range of calcium concentrations used (10—20 μM total calcium concentration). The change in the relative transmission is related to the change of absorption $\Delta I/I$ = —2 3 ΔA if $\Delta I/I << 1$. A similar calibration procedure with arsenazo III present but in the absence of rod outer segments yielded a change in the relative transmission of $\Delta I/I = 5.5 \cdot 10^{-2}$ A similar value $\Delta I/I$ = 5 3 10^{-2} was obtained by calculations under the assumption of a dissociation constant (K_D) of arsenazo III = 3 μM

Flash-spectrophotometry The principle and instrumental details of flash kinetic spectrophotometry have been reviewed elsewhere [31] The rod outer segment suspension was excited by a flash from a liquid dye laser (SUA 9, Electro-Photonics, Belfast) operated with coumarin 6 at 540 nm. The maximum output of the laser was about 30 mJ. The half-time of duration was 1 μs. To ensure reproducibility of the excitation energy, the laser was always operated at maximum electrical energy input (20 kV). For experiments where the calcium release was studied at various bleaching levels a frequency doubled YAG-laser (Laser Associates, output 10 mJ, half-rise time of duration 20 ns, output wavelength 530 nm) was employed. For both excitation sources the output energy was attenuated by neutral density filters (Wratten, Eastman Kodak) when lower bleaching levels were desired.

The intensity of the interrogating light source was 50—100 μW/cm^2 at 655 nm. The cuvette stayed in the flash-photometer 5—30 s before application of a flash to permit recovery from the pipetting step The recovery is indicated by a continous change of the steady scattering level until a constant value is reached During measurement in the flash-photometer no significant bleaching of the sample by the incident measuring light was observed.

Electrical signals were recorded DC under compensation of the dark DC-level. The time resolution was limited by the time-per-address setting of the averaging computer (ranging from 40 μs in Fig. 4A to 40 ms in Fig. 5A).

Results

Separation of the calcium-indicating absorption changes from responses to other events

Rod outer segment suspensions excited by a light flash exhibit true absorp-

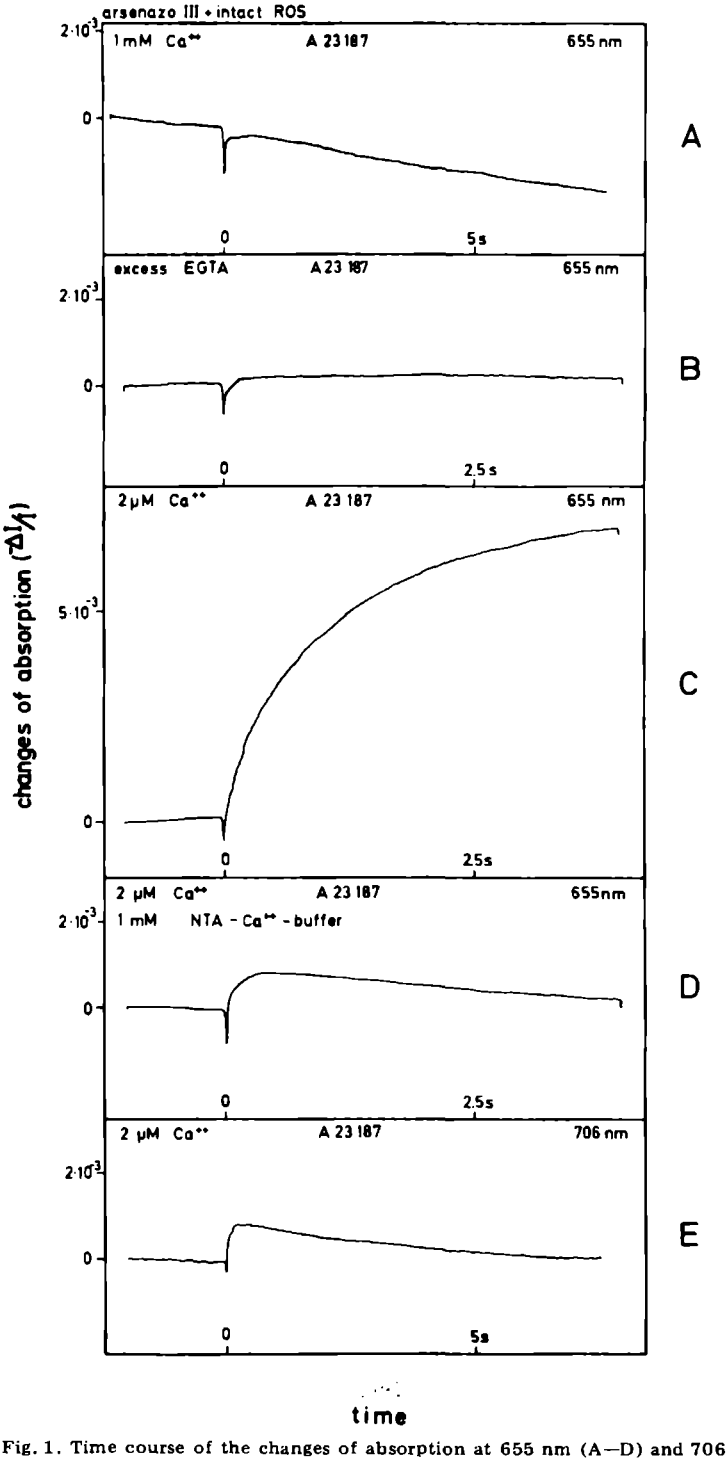

Fig. 1. Time course of the changes of absorption at 655 nm (A—D) and 706 nm (E) after excitation of a rod outer segment suspension by a flash at $t = 0$ in the presence of arsenazo III and ionophore A23187. Suspension medium: sucrose 600 mM, Ficoll 400 5%, Tris-HCl buffer 20 mM at pH 7.4, arsenazo III 30 μM, A 23187 10 μM. Rhodopsin concentration 4 μM. Bleaching level: 10% rhodopsin/flash. Flash energy 10 mJ. Excitation wavelength 530 nm. Temperature 20° C.

tion changes due to photolysis of rhodopsin and apparent absorption changes caused by transients of the light scattering properties [32] In the presence of arsenazo III, additional absorption changes are observed with intact rod outer segments but only when the ionophore A23187 is added (Fig 1C). These absorption changes have been monitored at 655 nm where underlying absorption changes resulting from photolysis of rhodopsin are negligible. The contribution of light-scattering transients was minimized by placing the absorption cell directly in front of the photomultiplier. Remaining apparent absorption changes due to changes of the light-scattering properties of the suspension can be isolated from the calcium-induced arsenazo III response by choosing conditions where this indicator is insensitive to small changes of the calcium concentration. The following controls were carried out·

(1) Very high (1 mM) and very low ($<10^{-7}$ M) calcium concentrations In contrast to the rather large absorption change observed at 655 nm when the calcium concentration was matched to the maximum of the sensitivity of arsenazo III ($pCa = pK_{ars}$, Fig. 1C) only minor responses were detected at very high and very low calcium concentrations (Fig. 1A and B) This clearly demonstrates that the arsenazo III response shown in Fig 1C is sensitive to the calcium level in the suspension. Furthermore Fig 1A and B show that at these calcium concentrations no apparent absorption change resulting from light-scattering can be detected.

(2) Measurement at the isosbestic point of arsenazo III at 706 nm Only a small absorption change is observed (Fig. 1E) when the measuring wavelength was adjusted to 706 nm, an isosbestic point of the calcium-arsenazo III complex. The size of the apparent absorption changes resulting from light-scattering at 706 nm is comparable to that at 655 nm [32].

(3) Suppression of the changes of the free calcium concentration by a calcium buffer In the presence of 1 mM nitrilotriacetic acid/calcium buffer adjusted to a free calcium concentration of 2 μM, a small flash-induced absorption change is observed (Fig. 1D). By comparison of Fig. 1C and D it is evident that the calcium-induced arsenazo III response is sensitive to the presence of a calcium buffer.

(4) Omission of ionophore A23187 in the reaction medium In the absence of A23187 in the medium no light-induced arsenazo III signal is observed (Fig. 2).

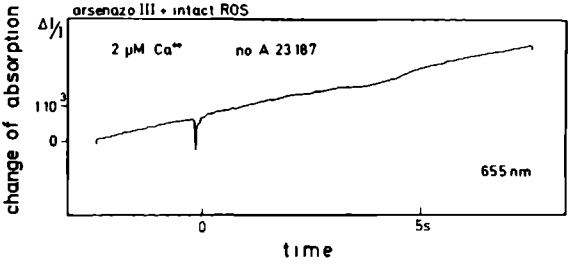

Fig 2 Time course of the change of absorption at 655 nm after excitation of a rod outer segment suspension by a flash at $t = 0$ in the presence of arsenazo III and without ionophore present Further conditions as in Fig 1C

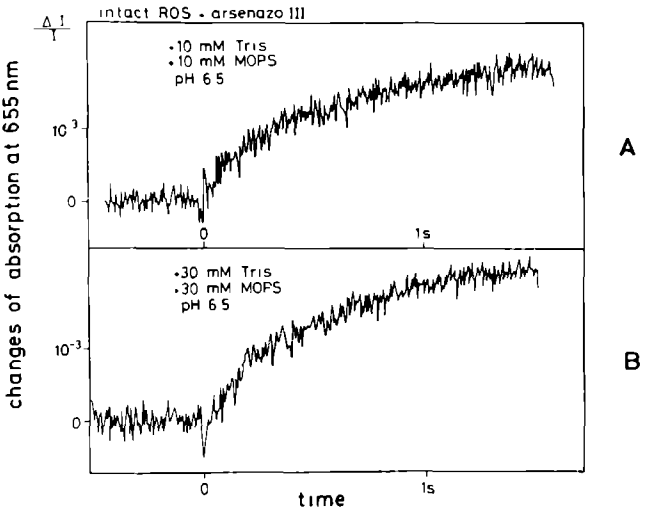

Fig. 3. Time course of the changes of absorption at 655 nm after excitation of a suspension of intact rod outer segments by a flash at $t = 0$. (a) Tris-HCl 10 mM, MOPS 10 mM at pH 6.5 (b) Tris-HCl 30 mM, MOPS 30 mM at pH 6.5. Rhodopsin concentration 5 μM. Bleaching level: 3% rhodopsin/flash. Free calcium concentration 2 μM.

It should be emphasized that for the experiments shown in Fig. 1D and E and in Fig. 2 where only minor flash-induced apparent absorption changes are observed, the calcium concentration was close to the maximum of the sensitivity of arsenazo III (pCa = pK).

The rapid net uptake of one proton/rhodopsin bleached during the metarhodopsin I/metarhodopsin II transition [33] represents another possible source of artifacts because arsenazo III is responsive to pH changes as well as to changes in calcium concentration [34]. The buffering capacity of the 20 mM Tris-HCl buffer is about 6 mM at pH 7.4, thus exceeding the maximal proton uptake of 5 μM (5 μM rhodopsin present) by a factor of thousand. Upon raising the proton buffering capacity threefold the arsenazo III response remains unchanged (Fig. 3). The adequacy of the proton buffering is not dependent on the buffering species. Imidazole, a permeating buffer, 2-(N-morpholino)-ethanesulfonic acid (MES) and morpholinopropane sulfonic acid (MOPS) gave similar results.

By choosing a higher time resolution a flash-induced absorption change of arsenazo III alone can be detected (Fig. 4A). When Fig. 4A and B are compared it can be seen that the flash-induced absorption change due to arsenazo III alone is fully reversible with a half-decay time of about 4 ms and thus does not interfere with measurements of the calcium-sensitive response shown in Fig. 1C, which was recorded at a much lower time resolution.

The foregoing experiments clearly demonstrate that calcium-indicating absorption changes of arsenazo III observed in rod outer segment suspensions are only slightly distorted by underlying absorption changes due to other events. Hence, in principle calcium fluxes in photoreceptor cells can be kinetically and quantitatively resolved.

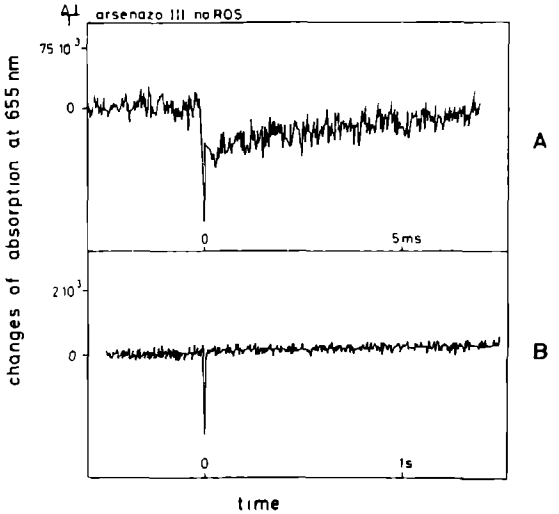

Fig. 4. Time course of the changes of absorption at 655 nm upon excitation of arsenazo III alone by a flash at $t = 0$. Arsenazo III 30 μM in standard medium at pH 7.4. Excitation wavelength 540 nm. Flash energy 4 mJ/cm^2. Free calcium concentration 2 μM.

Different rod outer segment preparations

In rod outer segments with an intact outer membrane light-evoked calcium release can be only detected in the presence of the ionophore A23187 (Fig. 5C). To permit discrimination between calcium release into the internal disc volume and calcium release into the cytoplasmic space two further preparations have been used. Leaky rods, with a plasma membrane permeable to small solutes behave in a way similar to intact rods: only in the presence of A23187 is calcium release observed (Fig. 5A). Second, in lysed rod outer segments, which have no outer membrane and where the disc stacking is disrupted, calcium release is almost completely abolished, even in the presence of A23187 (Fig. 5B).

Kinetics of light-induced calcium release

The rate at which calcium appears in the external medium (where arsenazo III is located) depends on the concentration of the carrier A23187 (Fig. 6). The rise of the calcium-indicating absorption changes at high ionophore concentrations ($\geqslant$10 μM) represents a minimal rate at which calcium is dissociated. It is of course possible that the actual dissociation rate is even faster.

Stoichiometry of the light-induced calcium release

One of the most important features of the calcium transmitter hypothesis is the high stoichiometry at which calcium must be released to give the observed electrophysiological responses [2,35,36]. In a recent estimate, 400—1000 Ca^{2+} released/rhodopsin molecule bleached are required at extremely low bleaching levels (a few photons absorbed/rod) [36]. For higher bleaching levels this high stoichiometry should decrease and finally saturate due to a depletion of calcium from discs.

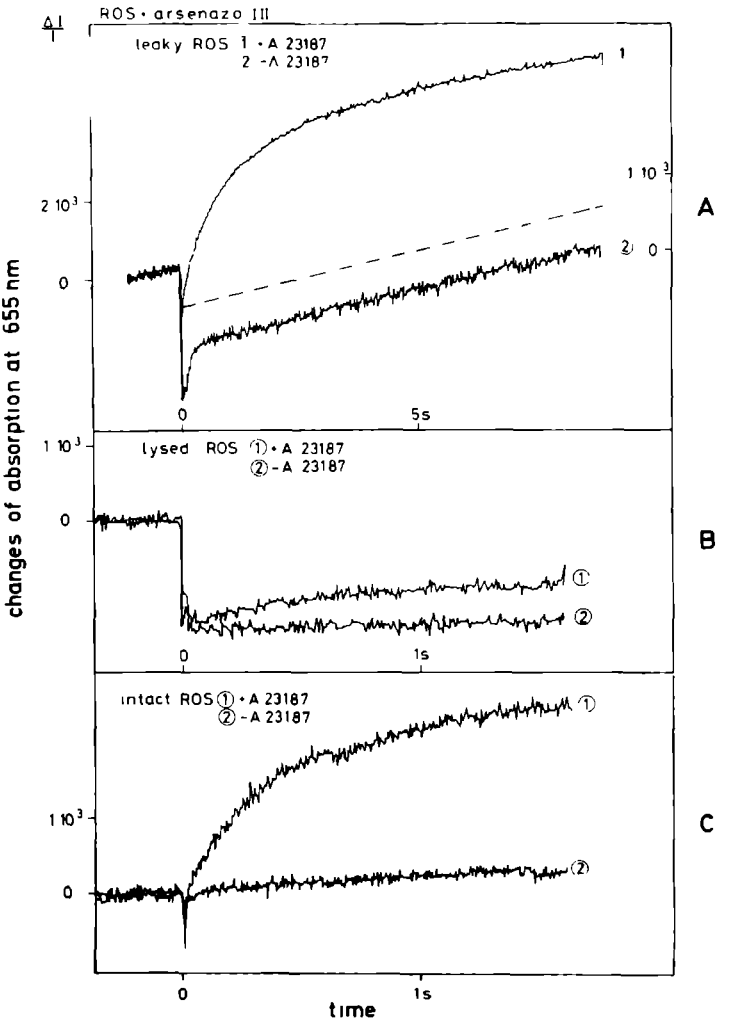

Fig. 5. Time course of the changes of absorption at 655 nm upon excitation of different suspensions of rod outer segments by a flash at $t = 0$ in the presence of arsenazo III. (a) leaky rod outer segments, bleaching level 10% rhodopsin/flash, rhodopsin concentration 5 μM. (b) lysed rod outer segments, bleaching level 10% rhodopsin/flash, rhodopsin concentration 5 μM. (c) intact rod outer segments, bleaching level 3% of the rhodopsin, rhodopsin concentration 5 μM. Free calcium concentration 2 μM. Traces 2 represent absorption changes after addition of 10 μM A 23187 to the suspension. Note that in Fig. 5A trace 2 is displayed two times more sensitive than trace 1. Dashed line represents the same display as in trace 1. Further conditions as in Fig. 1C.

We therefore measured the stoichiometry of the light-evoked calcium release, observed in this study, at various bleaching levels. When 1% to 10% of the rhodopsin is bleached, the calcium-indicating absorption changes at 655 nm (arsenazo III) are proportional to those recorded at 382 nm (metarhodopsin II) (Fig. 7). The stoichiometry observed amounted to 0.5 calcium ions released/rhodopsin molecule bleached at a free calcium concentration of 2 μM. The absorption changes at 382 nm were recorded separately but under identical

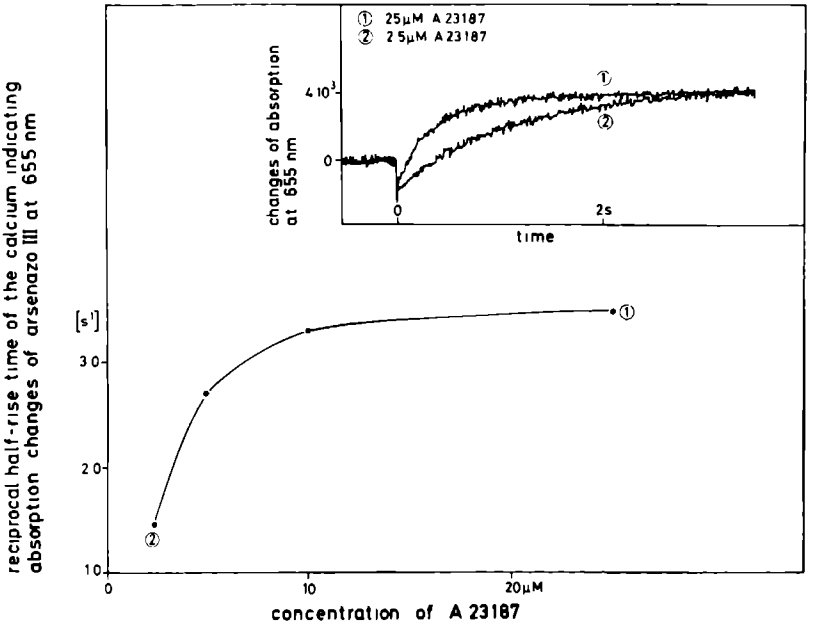

Fig. 6. Dependence of the half-rise time of the calcium-induced arsenazo III response on the concentration of ionophore A23187. Insert: time course of the changes of absorption at 655 nm upon excitation of a suspension of intact rod outer segments in the presence of arsenazo III and ionophore A23187. Concentration of A23187 as indicated. Bleaching level: 10% rhodopsin/flash. Further conditions as in Fig. 1C.

conditions to those under which the calcium-indicating absorption change of arsenazo III was measured: that is, in the presence of arsenazo III to include the filter action of the dye with respect to the exciting flash.

It should be noted that for all bleaching levels (down to 0.5%) the amplitude of the calcium-sensitive arsenazo III response was determined in single flash experiments.

Stability of the preparation with respect to the calcium release and reproducibility of the calcium-indicating arsenazo III responses

A study of the stability of the calcium storage system and a detailed analysis of the calcium fluxes in the dark have been described elsewhere for these preparations [26,27].

The light-evoked absorption changes due to release of calcium from binding sites are reproducible. Signals from different samples of the same preparation differ by at most 10%; signals from different preparations by at most 30%. In the course of our work more than 500 signals from about 12 preparations have been recorded. Storage of the stock suspension in the refrigerator for three days did not decrease the amplitude of the calcium response by more than 20%.

On the other hand, the release mechanism seems to be very sensitive to mechanical destruction of the disc stack structure. Mechanical stirring of the

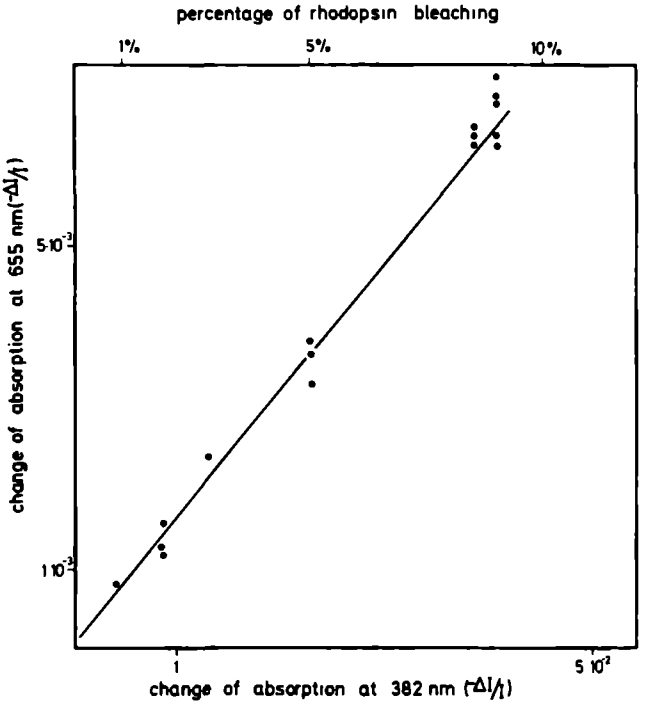

Fig. 7. Amplitudes of the absorption changes at 655 nm (arsenazo III) and at 382 nm (metarhodopsin II) for various bleaching levels. Conditions as in Fig. 1C.

suspension or the pellet during a resuspension step can abolish the release capacity almost completely. Also, after lysis of intact rod outer segments a calcium-induced arsenazo III response can no longer be observed. Finally, upon inclusion of electrolytes in the suspension medium the stability of the preparation, the calcium-indicating arsenazo III response and the calcium binding capacity are all affected [27].

Discussion

The above spectrophotometric studies with the metallochromic dye arsenazo III show that light-triggered calcium release by cattle rod outer segments can be optically detected and quantitatively and kinetically resolved. It has been demonstrated that optical signals resulting from responses of arsenazo III to other events and from intrinsic absorption changes of photoexcited rod outer segments do not interfere with the detection of the calcium release in the time range between 10 ms and 20 s. We have been able to resolve signals in single flash experiments resulting from photoexcitation of less than 1% of the rhodopsin.

In intact rod outer segments a rapid light-induced calcium release (<1 s) is only 'seen' by the external arsenazo III when the divalent cation ionophore A23187 is present. Surprisingly, in rod outer segments with a leaky outer membrane and in lysed rod outer segments also no fast light-induced calcium

release (<20 s) can be observed without ionophore. However, when the ionophore is present, leaky rod outer segments give a response similar to that observed in intact rod outer segments. Although we did not demonstrate the leakiness of the outer membrane for calcium ions directly, it seems reasonable to conclude that a membrane leaky to small solutes like ATP, NADPH and protons [26] is also leaky to calcium ions. However, the leakiness of the outer membrane for these solutes has been measured on a longer time scale than that used in our flash-spectrophotometric experiments with arsenazo III. Hence, a final conclusion on where calcium is released has to wait for kinetic data on the diffusion of calcium ions through the outer membrane of leaky rod outer segments.

The ionophore A23187 has been shown to reside in both disc and plasma membranes of intact rod outer segments [27]. This has been confirmed independently by the redistribution of calcium in intact rod outer segments upon addition of A23187 followed spectrophotometrically with arsenazo III. Therefore, the most attractive explanation of our findings in terms of the Yoshikami-Hagins hypothesis, namely that the ionophore functions exclusively in the outer membrane, thus communicating calcium, released from discs into the cytoplasma, across the envelope to the dye molecules in the external phase, is unwarranted.

Upon addition of A23187 to intact rod outer segments the free calcium concentration appears to be equilibrated between the intradiscal, cytosolic and external compartments [27]. The observation that calcium release in intact rod outer segments is seen only in the presence of the ionophore, suggests that the calcium ions were originally bound to the disc membranes rather than dissolved. The experiments with leaky and lysed rod outer segments further imply that the bound calcium pool, from which calcium is released, is exclusively located at the intradiscal membrane surface. It has been independently shown that in these preparations more than 90% of the endogenous calcium content of intact rod outer segments is stored at intradiscal binding sites [27]. From this it is concluded that the light-induced calcium-indicating arsenazo III response represents calcium release from intradiscal binding sites.

From the above it is concluded that probably no short-term control of the cytosolic calcium level by a light-evoked calcium release from the intradiscal calcium store occurs. This conclusion is consistent with those studies, which failed to detect a release from retinal discs [10,11,14,37]. However, it is difficult to compare our results to those studies which do report a light-triggered calcium release from discs [7,13,15,16,19—21]. This may be appreciated from the following aspects:

1. In the present study calcium release is abolished upon fragmentation of intact rod outer segments, whereas in other studies calcium release is observed in fragmented material [7,13,15,19,20].

2. We were unable to detect any calcium release in rod outer segments unless ionophore A23187 was added. In contrast Smith et al. [19] report that calcium release is completely abolished upon addition of A23187.

3. Smith et al. [19] only observe calcium release at high intradisc calcium concentrations (>5 mM) whereas in our experiments a comparable stoichiometry was obtained at a 1000-fold lower free calcium concentration (2 μM).

4. In most of the studies (but see Ref. 21) a half-rise time of the calcium

efflux from retinal discs on a minute scale was detected, whereas in this study it has been shown that calcium release from binding sites occurs with a half-rise time $\tau_{1/2} \leqslant 300$ ms

Light-induced calcium release from intradiscal binding sites is communicated to the arsenazo III in the external medium by the carrier A23187. Therefore, the observed rise time of the light-induced arsenazo III response determines an upper limit for the actual time required for calcium release from intradiscal binding sites Nevertheless, the time course of the calcium signals, observed in our study in the presence of A23187, is compatible with the rise-time of the photoreceptor potential or photovoltage at low bleaching levels [4,6,36] Therefore rapid calcium release from binding sites could throughout represent a trigger step in the transduction process. However, our results are not in agreement with the role of calcium as intracellular transmitter as formulated by Yoshikami and Hagins [1,2] unless two further assumptions are made (1) Some factor, required to mediate translocation of released calcium across the disc membrane is lost in isolated rod outer segments. (2) The linear stoichiometry of calcium released/rhodopsin bleached does not hold for very low bleaching levels at which the electrophysiological response saturates ($<<1\%$).

Our further efforts are directed to meet with the technical demands necessary to check the above mentioned assumptions. Alternative functions of the described effects are also under investigation

Acknowledgements

We wish to thank Ms I. Columbus for competent technical assistance and A McGeer (M Sc), Dr H -W Trissl and F.J M. Daemen for reading the manuscript and helpful discussions One of us (P.S) was recipient of a travel grant from the University of Nijmegen (The Netherlands) and of a short-term fellowship from EMBO.

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Biochimica et Biophysica Acta, 554 (1979) 441—459

BBA 78414

CALCIUM TRANSLOCATION AND STORAGE OF ISOLATED INTACT CATTLE ROD OUTER SEGMENTS IN DARKNESS

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(Received November 22nd, 1978)

Key words. Ca^{2+} exchange, Ca^{2+} storage, (Rod outer segment)

Summary

Bovine rod outer segments (rods), isolated with an intact plasma membrane and a stable calcium exchange and storage capacity, contain 2—3 mol endogenous calcium/mol rhodopsin. By means of ^{45}Ca accumulation experiments and concomitant ^{40}Ca analysis, the calcium metabolism of these organelles has been studied with the following results:

1. The majority of endogenous calcium is localized within disks.
2. In the presence of the ionophore A23187 the intradiskal binding sites can be titrated with external calcium.
3. The Scatchard plot of calcium binding of rods indicates the presence of a single set of intradiskal binding sites with a maximal capacity of 8—9 mol calcium/mol rhodopsin and an affinity constant of 55 μM to calcium.
4. Without A23187 more than 99% of the rod calcium appears in a bound state in equilibrium with a free calcium concentration of 15—25 μM.
5. External calcium exchanges with endogenous calcium in a fast ($t_{1/2}$ = 12 s) process with a uniform rate constant, whereas net calcium transport is very slow ($t_{1/2} > 2$ h).
6. Intact rods contain a calcium translocation system, presumably located in the plasma membrane, which performs Ca-Ca exchange with a high unidirectional flux of $2 \cdot 10^6$ calcium ions/rod per s.
7. This translocation system can be saturated by external calcium (K_m = 0.5—1 μM) and has a low Q_{10} (1.08).

Both the calcium translocation system and the calcium binding system appear to depend on the structural integrity of the stacked disks and are very sensitive to the experimental conditions.

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The relevance of these findings is discussed in relation to the proposed role of calcium ions as the intracellular transmitter in vertebrate rod photoreceptor cells.

Introduction

Since Hagins (1) proposed Ca^{2+} as the intracellular transmitter in vertebrate rod outer segments (rods), a number of reports have appeared [2—5], which describe the endogenous calcium content of isolated rods and the effect of illumination on it. In another series of studies [6—14], rod fragments are loaded with exogenous calcium and a possible subsequent light-induced release is investigated. However, in both cases serious discrepancies are obvious. The calcium content may very over almost two orders of magnitude. With respect to a light-induced Ca release from rod disks, disagreement is even greater.

In view of this confusing situation we have focussed our attention on calcium storage and transport of isolated rods in darkness [15]. In a previous report [16] we established that cattle rods with a leaky plasma membrane contain a specific calcium translocation system in the disk membrane, sensitive towards Ca^{2+} and Na^{+}, but not to Mg^{2+} and K^{+}. In vitro this system does not behave as a (Ca^{2+} + Mg^{2+})-activated ATPase calcium pump, but under certain conditions it can be affected by ATP, resulting in net calcium uptake. The major ATP-independent transport modes are Ca-Ca exchange and Na-stimulated Ca efflux, presumably by Na-Ca exchange.

In addition to the translocation system cattle rod membranes also possess a calcium storage capacity, dependent on the structural integrity of the intact stack of disks and possibly depending on or generated by the hydrolysis of ATP [16]. The molecular basis of this storage capacity might be related to the presence of calcium binding sites on rod membranes, described by Hendriks et al. [17] and Hemminki [18].

Since the previously used rod preparations appeared to have a leaky plasma membrane and to be very labile in various respects [16], we have devised a new isolation procedure [19]. This yields cattle rods with an intact plasma membrane, in which the calcium translocation and storage systems are much better preserved. Moreover, the intactness of their plasma membranes can conveniently be manipulated without harming their stability. In this paper, some basic features of calcium translocation and storage in these rod preparations are described.

Materials and Methods

Standard procedures

All procedures with rods are carried out in darkness or in dim red light. ^{45}Ca translocation into rods is assayed by the rapid filtration method described before [16]. Throughout all experiments the washing medium contains 600 mM sucrose, 20 mM Tris-HCl (pH 7.4) and 250 μM EGTA, which removes all adherent Ca. Radioactivity is counted in 10 ml Aquasol (New England Nuclear, Boston, U.S.A.) in a liquid scintillation counter.

Calcium contaminations in the media are reduced by passing all sucrose and sucrose-Ficoll solutions over a mixed-bed ion-exchange column. The resulting calcium contamination is less than 1 μM. Tris (250 mM) does not contain a measurable calcium contamination, LiCl contains 15 μM calcium/100 mM LiCl and KCl contains 0.5 μM or 2 μM calcium/100 mM KCl (two different lots of KCl).

Rhodopsin determinations are performed according to the standard procedures of this laboratory [20].

Preparations

Three types of rod preparation have been used in this study. Stable intact rods are prepared in a sucrose-Ficoll medium according to the previous paper [19]. Their plasma membrane is intact, since it constitutes a permeability barrier for ATP, NADPH and protons. Whenever the term 'rods' or 'rod outer segments' is used in this paper without further qualification, it refers to this preparation.

Stable leaky rods are isolated in 0.16 M Tris-HCl buffer (pH 7.4) and finally resuspended in the sucrose-Ficoll medium. They lack the permeability barrier for ATP, NADPH and protons [19]. Both these preparations can be stored at 4°C as a concentrated suspension (100 μM rhodopsin). In the experiments rhodopsin concentrations between 10 and 20 μM have been used.

Finally rods, isolated according to the old procedure [16] in 0.16 M Tris-HCl buffer (pH 7.4) and resuspended in electrolyte media, have been used. These 'Tris-rods' are leaky, as measured by the criteria mentioned above [16]. The prefix 'depleted' (as opposed to non-depleted) refers to preparations, depleted of endogenous calcium having EGTA present throughout the isolation procedure.

^{40}Ca determination

Calcium is determined with a Pye Unicam SP 1950 double-beam atomic absorption spectrophotometer. All samples contain 0.5% $LaCl_3$ to overcome anionic interference. Calcium standard solutions are prepared from anhydrous $CaCO_3$, which is dissolved in 0.1 M HCl containing 0.5% $LaCl_3$.

In view of the conflicting results on the calcium content of rod outer segments between different investigators, three different methods of sample preparation have been compared. In the dry ashing method an aliquot (0.5 ml) of a rod suspension is placed in a quartz tube (10 × 80 mm), dried at 75°C and ashed at 520°C for 6 h. The residue is heated with 30% H_2O_2 to boiling until a clear and colorless solution is obtained. This is dried at 75°C and dissolved in 0.1 M HCl containing 0.5% $LaCl_3$. In the ionophore extraction method an aliquot (0.5 ml) of a rod suspension is incubated at 18—20°C for 15 min with 4 μM A23187 (gift of E. Lilly and Co., Indianapolis, U.S.A.) and excess EDTA. Ionophore is added as an ethanolic solution. The suspension is centrifuged and calcium is determined in an aliquot of the clear supernatant, using standards prepared in the same medium. The calcium content of the sediment remaining after ionophore treatment is below the detection limit. Finally, the previously described [2] acid digestion method has been used. The calcium contents of three rod outer segment preparations were independently determined by each

method in 4—8-fold. If the value obtained by the ionophore extraction method is set at 100%, the dry ashing method yields 98.2% and the acid digestion method 99.1%, with standard deviations of 1.9, 2.9 and 2.4%, respectively. Since these values are equal within the experimental error, the ionophore method has been used routinely in all further experiments in view of its convenience.

Results

Incorporation of ^{45}Ca in intact cattle rods

The average total calcium content of the rod pellet, immediately after the gradient procedure, is given in Table I. After subsequent resuspension of the rods in the virtually Ca-free standard medium (600 mM sucrose/5% Ficoll 400/20 mM Tris-HCl, pH 7.4) ^{45}Ca is added to the rod suspension. After equilibration a considerable fraction (50—60%) of the total ^{45}Ca is present in the rods, as determined by the rapid washing/filtration procedure (Table I).

If external ^{45}Ca is separated from the incorporated ^{45}Ca by passing the suspension over a Sephadex G-50 column, more than 90% of the radioactivity eluted with the rods remains on the filter after the washing/filtration procedure. This shows that nearly all rods are retained by the filter and that only few rods are disrupted by the washing/filtration procedure.

Excess EGTA, which buffers the external, free calcium concentration to below 10^{-2} μM, abolishes the uptake of ^{45}Ca (Fig. 1). La^{3+} (50 μM) apparently greatly decreases the translocation rate of calcium, but has no influence on the final equilibrium level (Fig. 1). These effects demonstrate that the washing/filtration procedure with 250 μM EGTA removes all adherent ^{45}Ca and ensures that translocation into the rods, rather than binding to the plasma membrane is measured.

Since the plasma membrane of our rod preparations constitutes a permeability barrier to ATP, NADPH and protons [19] ^{45}Ca must have been translocated through the plasma membrane. In view of the high rate of equilibration of ^{45}Ca (Fig. 1; $t_{1/2}$ = 12 s; S.E. 1; n = 13), this membrane appears to contain a highly active calcium translocation system.

Exogenous ^{45}Ca exchanges with at least 90% of the endogenous Ca. This has been determined in two ways.

TABLE I

CALCIUM CONTENT OF ISOLATED INTACT RODS

The endogenous calcium content of the rods is calculated by multiplying the total calcium content of the rod suspension with the fraction of the total radioactivity, incorporated in the rods after complete equilibration.

	Standard procedure	1 mM $CaCl_2$ omitted in isolation medium
Total calcium content (mol Ca/mol rhodopsin)	5.7 ± 0.6 (11)	3.2 ± 0.3 (23)
Percent of added ^{45}Ca incorporated after equilibration	59 ± 5 (10)	55 ± 2.5 (19)
Endogenous calcium content (mol Ca/mol rhodopsin)	3.4 ± 0.4 (10)	1.8 ± 0.2 (15)

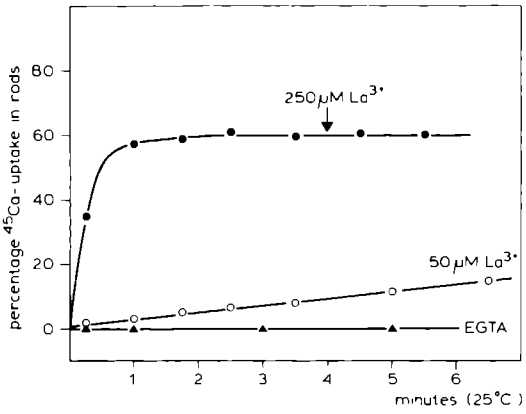

Fig. 1. Equilibration of ^{45}Ca between endogenous and exogenous calcium pools. Temperature: 25°C. Aliquots of the rod suspension are filtered over borosilicate glass filters. The radioactivity of the sediments is determined and expressed as percent of the total ^{45}Ca added. Medium: 600 mM sucrose, 5% w/v Ficoll 400, 20 mM Tris-HCl (pH 7.4). Triangles: in the presence of large excess EGTA (0.2 mM). Open circles: in the presence of 50 μM $LaCl_3$. Control level is ultimately reached. Closed circles: no additions at start, but after 4 min addition of 250 μM $LaCl_3$.

After addition of ^{45}Ca to a rod suspension, followed by equilibration and centrifugation, the ^{45}Ca/^{40}Ca ratios in supernatant and pellet differ by at most 10%. Addition of increasing amounts of ^{40}Ca decreases the ^{45}Ca incorporation proportionally to the decrease of the specific radioactivity as expected from a completely exchangeable calcium pool within the rods.

Reduction of external, free calcium by titration with EGTA causes changes in the rate of ^{45}Ca-^{40}Ca exchange (see Fig. 4 and its discussion) which allow an estimation of the initial external, free calcium concentration. The resulting values agree with those calculated from the ^{45}Ca distribution after equilibration and the total ^{40}Ca content of the suspension. Therefore, the endogenous calcium content of the rods can be obtained in good approximation by multiplying the total calcium content of the rod suspension with the fraction of the total radioactivity incorporated in the rods after equilibration (Table I).

When a rod suspension after the initial isolation is stored for varying times (up to 2 days at 4°C) before performing the ^{45}Ca uptake experiment, nearly the same ^{45}Ca uptake curve is observed as shown in Fig. 1. The final ^{45}Ca distribution between rods and external medium thus seems to be a very stable feature, despite the fact that the uptake of ^{45}Ca in exchange for endogenous ^{40}Ca is a very dynamic process.

Localization of endogenous calcium

In the previous paragraph it has been shown that exogenous ^{45}Ca exchanges with nearly all endogenous calcium in rods, resulting in a homogeneous distribution of ^{40}Ca and ^{45}Ca. Therefore, endogenous calcium can be quantitatively localized from the ^{45}Ca distribution after previous equilibration.

When leaky rods or lysed rods (disks) are used, the EGTA washing procedure is expected to remove all calcium except that which is located inside the disks [16]. An attempt to localize the calcium in intact rods is displayed in Fig. 2.

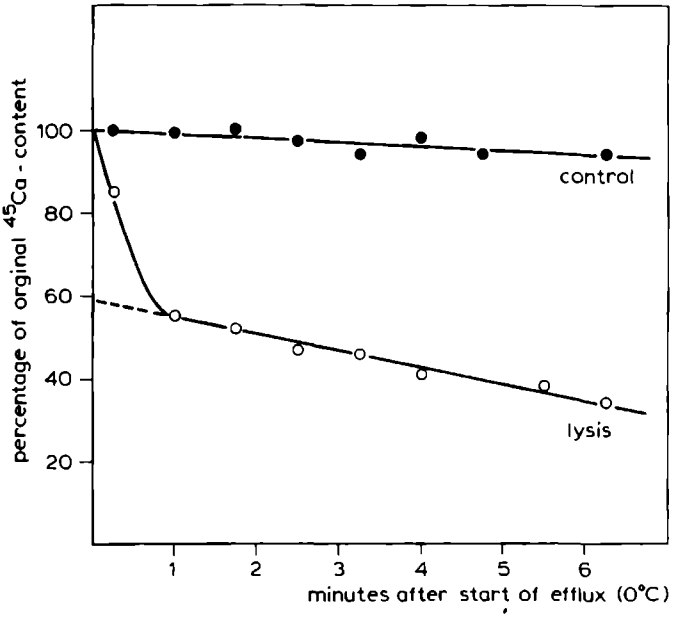

Fig. 2. Efflux of ^{45}Ca from pre-loaded rods in the presence of excess EGTA at 0°C. Rods, previously equilibrated with ^{45}Ca at 25°C, are ten-fold diluted with the indicated media. The data are expressed as percent with respect to the ^{45}Ca level after the previous equilibration. Closed symbols: control efflux in original medium: 600 mM sucrose, 5% w/v Ficoll 400, 0.2 mM EGTA, 20 mM Tris-HCl (pH 7.4). Open symbols: efflux after lysis in 5% w/v Ficoll 400, 0.2 mM EGTA. Results of three experiments with different preparations are averaged. The excess EGTA is sufficient to prevent re-uptake of ^{45}Ca.

Preloaded intact rods lose very little radioactivity upon dilution in an isosmotic, tracer-free medium containing excess EGTA. A ten-fold reduction in osmotic strength lyses the rods, but still at least 60% (extrapolation to zero time from the slow efflux phase) of the calcium remains inaccessible to EGTA and must, therefore, be located within the disks. The other 40% are lost in a fast efflux phase (Fig. 2), possibly due to rupture of part of the disks before or during the filtration procedure. This suggestion is strengthened by the observation that the contribution of the fast efflux phase strongly depends on slight variations of the conditions. Omission of Ficoll 400 from the lysis medium increases the fast efflux phase to 58%, whereas replacement of EGTA by La^{3+} (250 μM) in the lysis medium lowers it to 28%.

In agreement with these data obtained by ^{45}Ca analysis, the disk pellet after lysis (conditions as in Fig. 2) and subsequent centrifugation still contains about 80% of the ^{40}Ca, present in the non-lysed control. The 20% loss can roughly be accounted for by the slow efflux phase in view of the time elapsed between lysis and centrifugation.

These data indicate an exchange between external and intradiskal calcium. The participation of the calcium in the rod cytosol in this exchange is difficult to evaluate.

Properties of the calcium translocation system

Data on the net transport of calcium into rods after previous ^{45}Ca equilibra-

tion are summarized in Fig. 3. The half-times of net uptake (7 h with 1 mM Ca^{2+} outside) and net efflux (3 h with excess EGTA outside) are about three orders of magnitude slower than ^{45}Ca equilibration by exchange (Fig. 3 as compared to Fig. 1). This implies that the transport mode of the rapid calcium translocation system is a 1 : 1 exchange. Therefore, ^{45}Ca equilibration can be formally described by two opposing first order reactions. The unidirectional Ca flux of the exchange process can thus be determined by a kinetic analysis of ^{45}Ca equilibration. The appropriate equations are given in the legends of Fig. 4. The maximal unidirectional Ca flux, thus calculated, is surprisingly high and amounts to 3.85 mol calcium/mol rhodopsin per min (S.E. = 0.45, n = 15).

Titration of extracellular calcium with EGTA hardly affects the equilibration rate, until EGTA is in molar excess over total extracellular calcium. This indicates that the transport site is completely saturated with calcium at micromolar external calcium concentrations. With different amounts of EGTA, in excess over external calcium, various low free external calcium concentrations can be obtained. When ^{45}Ca uptake is restricted to a few minutes, no net calcium transport occurs and the data can be properly analyzed (Fig. 4). Free calcium concentrations are calculated according to Caldwell [21]. The linearity observed in these plots indicates a good homogeneity of the preparation, since all endoge-

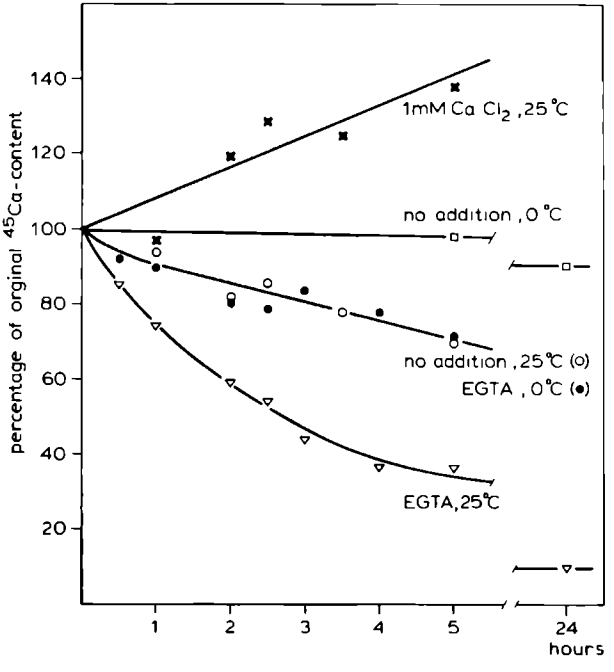

Fig. 3. Net transport in rods, previously equilibrated with ^{45}Ca. Medium: 600 mM sucrose, 5% w/v Ficoll 400, 20 mM Tris-HCl (pH 7.4). The data are expressed as percent with respect to the ^{45}Ca level after the previous equilibration. Crosses (X): 1 mM $CaCl_2$ added, temperature 25°C (previous equilibration in the presence of 1 mM $CaCl_2$ to adjust the specific radioactivity). Squares (□): no addition, temperature 0°C. Open circles (○): no addition, temperature 25°C. Closed circles (●): excess EGTA added (0.2 mM), temperature 0°C. Triangles (▽): excess EGTA added (0.2 mM), temperature 25°C. The excess EGTA (0.2 mM) is sufficient to prevent re-uptake of ^{45}Ca.

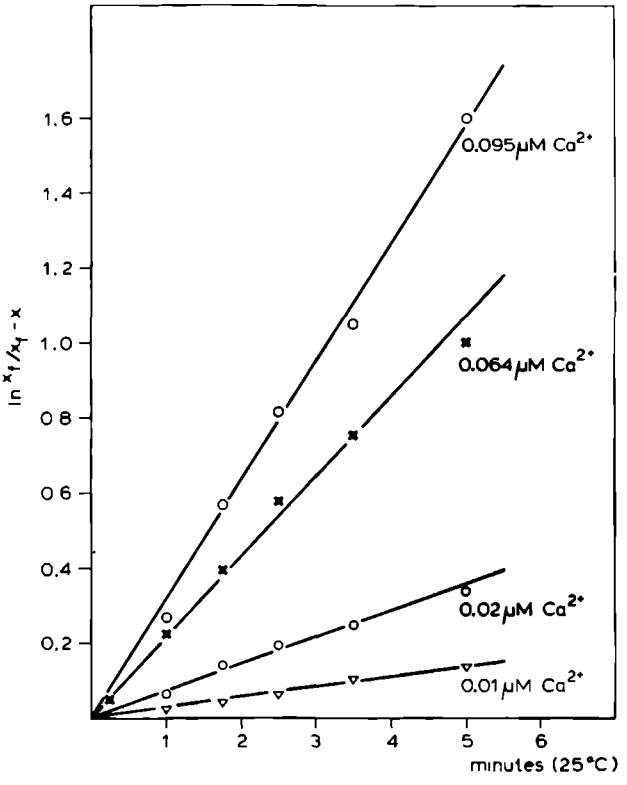

Fig. 4. Kinetic analysis of unidirectional ^{45}Ca fluxes. ^{45}Ca equilibration without net calcium transport can formally be described by two opposing first-order reactions, resulting in the equation: $\ln[x_f/(x_f - x)] = [k/x_f] \cdot t$, where x is the fraction of ^{45}Ca in the rods at time t, x_f is the fraction of ^{45}Ca in rods after equilibration, and k is the rate constant. The unidirectional flux is: $v = k(1 - x_f)Ca_t$, where Ca_t is the total calcium concentration, expressed as mol calcium/mol rhodopsin. Low free calcium concentrations are obtained by addition of various concentrations of EGTA, in excess over external calcium. Open circles: 0.095 μM Ca^{2+}; crosses: 0.064 μM Ca^{2+}, closed circles: 0.02 μM Ca^{2+}, triangles: 0.01 μM Ca^{2+}. The medium further contains 120 mM sucrose, 1.25% w/v Ficoll 400, 160 mM KCl, 20 mM Tris-HCl (pH 7.4). Temperature 25° C.

nous calcium pools exchange with the same rate constant.

The relation between external, free calcium concentration and the unidirectional ^{45}Ca fluxes can be further analyzed by transformation to a Lineweaver-Burk plot (Fig. 5). These plots appear to be linear from 0.01 μM Ca^{2+} to 10 μM Ca^{2+} (the latter is used as concentration with maximum velocity) and indicate saturation of a single type of transport site. The affinity of this transport site for calcium calculated according to this model and using the data points of Fig. 5 is 1.02 μM (S.E. 0.14; $n = 6$). Substitution of a major part of the sucrose by KCl does not greatly affect the result. The slightly higher apparent affinity for calcium in this case (0.52 ± 0.05 μM; $n = 5$) may be due to the effect of the electrolyte KCl on the association constants of EGTA. This effect may be comparable to the influence of various buffers [22].

The energetics of the Ca-Ca exchange has been investigated by determining its temperature dependence (Fig. 6). A Q_{10} value of 1.08 is found, indicating a heat of activation of only 1.3 kcal/mol. The Ca-Ca exchange can be maintained

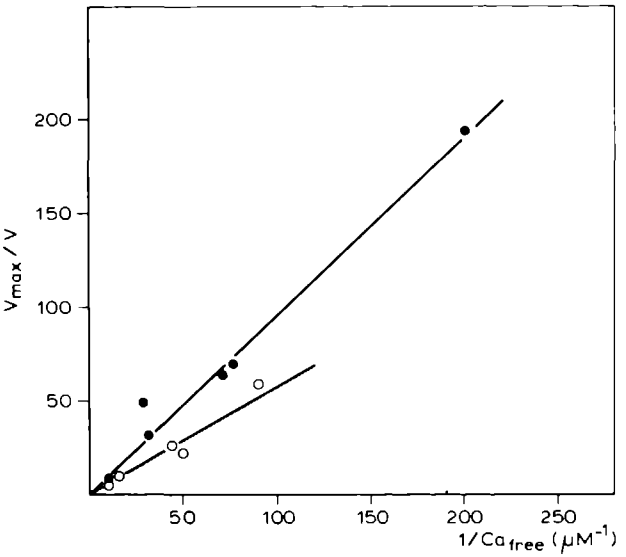

Fig. 5. Lineweaver-Burk plots of unidirectional ^{45}Ca fluxes. Open circles: data calculated from those in Fig. 4. Closed circles: same conditions as in Fig. 4, except for the medium: 600 mM sucrose, 5% Ficoll 400, 20 mM Tris-HCl (pH 7.4).

for a considerable length of time (several days at 4°C) without addition of exogenous energy sources.

With stable leaky rods [19] the unidirectional Ca flux of the Ca-Ca exchange is equal to that for intact rods within a factor of 2, and the kinetic analysis

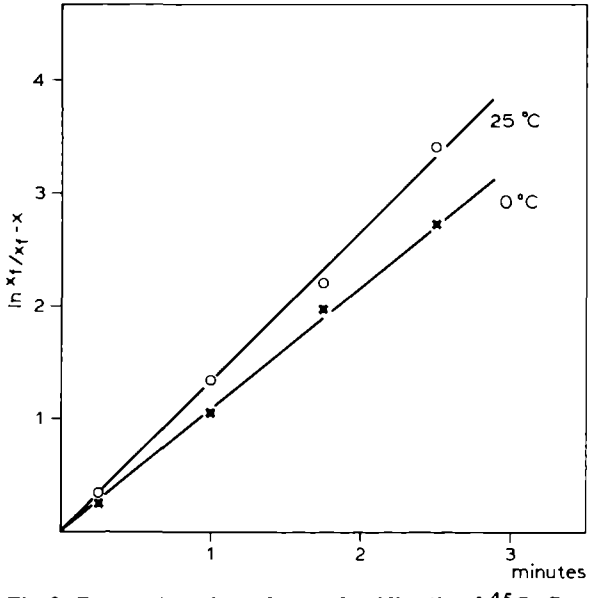

Fig. 6. Temperature dependence of unidirectional ^{45}Ca fluxes. The kinetic analysis is similar to that in Fig. 4. Open circles: 25°C, closed circles: 0°C. Medium: 600 mM sucrose, 5% w/v Ficoll 400, 50 mM KCl, 20 mM Tris-HCl (pH 7.4). The external calcium concentration (20 μM) is sufficient to saturate the transport system.

again yields linear plots. In the leaky 'non-depleted Tris rods' [16] ^{45}Ca equilibration by Ca-Ca exchange also takes place, but the unidirectional Ca flux of Ca-Ca exchange amounts to only 0.37 mol Ca^{2+}/mol rhodopsin per min (S.E. = 0.12, $n = 6$). This is a remarkable finding, since this flux is only 10% of that in intact rods, where calcium first has to pass the plasma membrane. The other properties of the Ca-Ca exchange in leaky 'Tris rods' are difficult to assess, because the kinetic analysis generally does not yield linear plots.

Properties of the calcium storage system

Stable intact rods lose only a small part of their endogenous calcium during storage at 4°C for up to 2 days (cf. Table II), while net efflux of calcium from rods down a calcium gradient (in the presence of 0.2 mM EGTA) has half-times in the order of hours, even at 25°C (Fig. 3). Therefore, as previously noticed for leaky Tris rods [16], the calcium content of intact rods appears to be virtually independent of the external calcium concentration.

When the divalent cation ionophore A23187 is added to the suspension, the calcium content of the rod becomes dependent on the external calcium concentration (Fig. 7A). The resulting linear Scatchard plot (Fig. 7B) suggests that a single set of binding sites is titrated with calcium. The maximal capacity of these sites amounts to 8—9 mol Ca^{2+}/mol rhodopsin and their affinity constant for calcium is 55 μM. The ionophore A23187 makes both the plasma membrane and the disk membrane fully permeable to calcium because endogenous calcium located within the disks becomes accessible to rapid complexation by EGTA (Fig. 8). When, after prior depletion, calcium is added back to the

TABLE II

EFFECTS OF VARIOUS MEDIA ON THE CALCIUM CONTENT AND ON THE Ca-Ca EXCHANGE

Results are presented as percent with respect to the calcium content and translocation rate of the rods in the standard medium. The calcium content is determined at 25°C by the ^{45}Ca level in the rods after equilibration (cf. Table I). The translocation rate is the maximal velocity of the unidirectional Ca flux of Ca-Ca exchange at 25°C. The half-time of ^{45}Ca-equilibration is the time required to reach half of the equilibrium ^{45}Ca level in rods at 25°C. It can be calculated with the equation given in the legend of Fig. 4. The calcium content in the presence of EGTA is determined after previous equilibration of ^{45}Ca at 25°C. The EGTA concentration is sufficient to prevent uptake of ^{45}Ca.

Medium	Storage conditions:						
	Calcium content				Calcium translocation rate		
	0 h	24 h 4°C	5 h 4°C + 0.2 mM EGTA	5 h 25°C + 0.2 mM EGTA	0 h	24 h 4°C	0 h $t_{1/2}$ of equilibration
Standard sucrose-Ficoll medium	≡100%	91	70	34	≡100%	95	≡1 (12 s)
KCl *	90	37	42	34	66	69	1.7
LiCl *	90	56	62	12	20	5	8.0
Tris-HCl *	76	24	n.d.	n.d.	35	11	3.2
KCl **	68	9	n.d.	n.d.	75	7	1.2

* 200 mM KCl, 200 mM LiCl, 230 mM Tris-HCl, respectively, with 20 mM Tris-HCl (pH 7.4) added, substitute 80% of the standard medium.

** Pellet resuspended in 200 mM KCl, 20 mM Tris-HCl (pH 7.4).

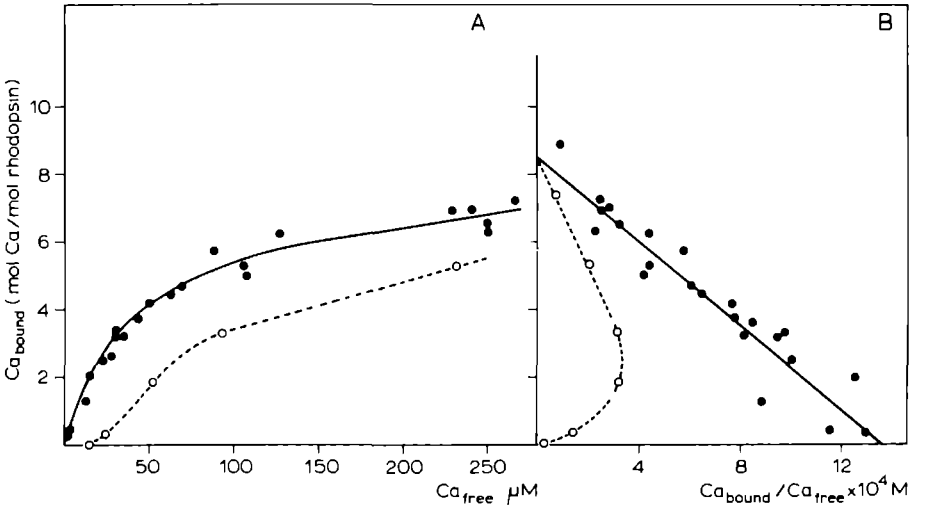

Fig. 7. Calcium binding by rods in the presence of A23187. Medium 600 mM sucrose, 5% w/v Ficoll 400, 20 mM Tris-HCl (pH 7.4). A. Rods are equilibrated for 45 min at 25°C with ^{45}Ca in the presence of various amounts of added ^{40}Ca and EGTA (to obtain the lower calcium concentrations by reduction of endogenous calcium).Closed circles· Results of five different rod preparations (A23187 concentrations between 0.15 and 0.40 mol A23187/mol rhodopsin). Open circles Rods are preincubated for 30 min at 25°C with 100 mM NaCl (replacing 200 mM sucrose) and excess EGTA (1 mM) After removal of NaCl and EGTA, the ionophore is added (0.2 mol A23187/mol rhodopsin) The further procedure is similar to that without preincubation. B. Scatchard plots from data of A. Same symbols used as in B

medium in a concentration range of 5—400 μM free calcium, calcium is bound again to the intradiskal binding sites to almost the same values (102%, S.E. = 4, n = 18) as before depletion.

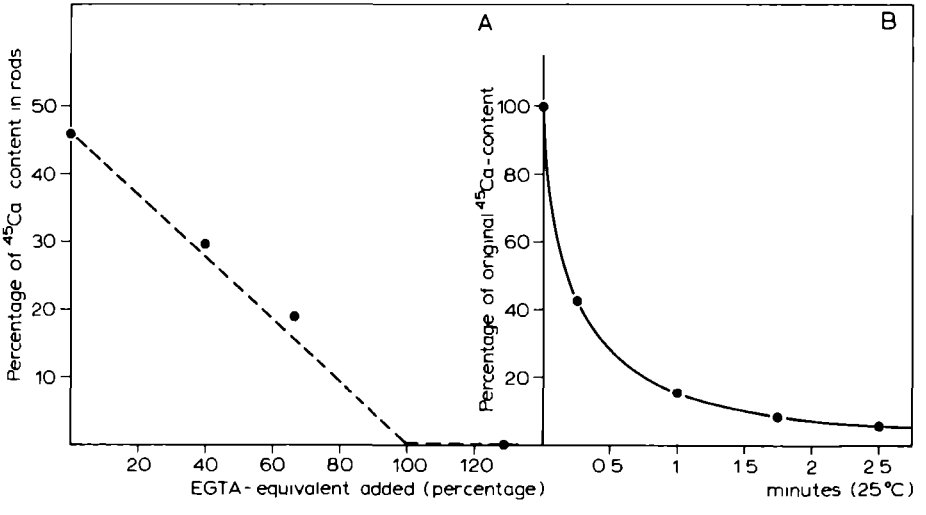

Fig. 8. Titration of ^{45}Ca from rods by EGTA. Medium 600 mM sucrose, 5% w/v Ficoll 400, 20 mM Tris-HCl, pH 7.4. Temperature 25°C. A. Rods are equilibrated with ^{45}Ca and various amounts of EGTA in the presence of A23187 (0.2 mol A23187/mol rhodopsin). EGTA-equivalent is expressed as percent of the total amount of calcium present. B. ^{45}Ca-efflux from rods, previously equilibrated with ^{45}Ca in the presence of A23187 (0.1 mol A23187/mol rhodopsin), by addition of excess EGTA (0.2 mM).

The Scatchard plot of calcium binding may show small deviations of linearity, because the optimal ionophore concentration varies somewhat with the free calcium concentration (not shown). Consistent deviations from linearity in the Scatchard plot may be observed upon isolation of rods in a low calcium medium (in the presence of EGTA). Upon exposure of rods to a medium containing EGTA and high Na^+, these deviations become more pronounced. The most extreme case observed is included in Fig. 7 (broken line) and shows a sigmoidal relation between Ca binding and the external calcium concentration.

Stability of the calcium storage and translocation system

The stability of both the calcium storage and translocation systems of rods in the sucrose-Ficoll medium contrasts with the observations for the leaky Tris rods [15], where electrolyte media were applied. Therefore the effect of partial medium substitution by various electrolytes has been investigated. NaCl has strong direct effects on Ca metabolism in isolated cattle rods, which will be presented in a subsequent communication and hence are omitted here.

When 80% of the sucrose-Ficoll medium is substituted by various electrolyte media, the rod outer membrane remains intact (no phosphorylation by exogenous ATP) and the Ca translocation and storage characteristics of the rods are only slightly affected (Table II). However, exposure to these media for some hours appears to decrease the stability of the rods more seriously (Table II). Tris and lithium ions affect both the Ca translocation rate and the calcium content (storage capacity), while K^+ mainly affects the latter. By resuspension of a pellet of intact rods in the indicated electrolyte media, the rods become leaky [19] and are much less stable (Tables III and V).

Table III shows the half-time of net ^{45}Ca efflux against excess external EGTA for various rod preparations. Net efflux increases by a factor of 35 going from stable rods with intact or leaky plasma membranes to the most maltreated rods: freeze-thawed depleted Tris rods. Concomitantly, the maximal velocity of the exchange system and also the sensitivity to La^{3+} are greatly decreased. Table IV demonstrates that the strong inhibition of Ca-Ca exchange by La^{3+} is gradually abolished in this series. This is not due to the fact that in the one case transport through the plasma membrane and in the other through the disk membrane is measured, since La^{3+} can also inhibit Ca-Ca exchange in lysed and leaky rods.

TABLE III

HALF-TIME OF ^{45}Ca EFFLUX AGAINST EXCESS EGTA FOR VARIOUS ROD PREPARATIONS

Preparation	Half-time (min; 25°C)
Intact rods in sucrose-Ficoll medium	150—180
Intact rods, 200 mM KCl substitutes 80% of sucrose-Ficoll medium	150
"Non-depleted Tris rods" * in K-medium (100 mM KCl, 2 mM $MgCl_2$, 20 mM Tris-HCl, pH = 7.4)	40— 50
"Depleted Tris rods" * in K-medium	10— 15
"Depleted Tris rods" * in K-medium after freeze-thawing	4— 6

* These preparations are defined under Materials and Methods and were used in a previous study [16].

TABLE IV

EFFECT OF LANTHANUM ON ^{45}Ca TRANSLOCATION IN VARIOUS ROD PREPARATIONS

^{45}Ca uptake after 1 min at 25°C in the presence of 100 μM La^{3+}, expressed as percent of uptake in a control suspension without La^{3+}. External calcium concentration: 15—30 μM.

Preparation	Percent ^{45}Ca uptake	State of the plasma membrane
Intact rods in sucrose-Ficoll medium	10	intact
Intact rods, 200 mM KCl substituting 80% of sucrose-Ficoll medium	11	intact
Lysed rods in sucrose-Ficoll medium	2	absent
Intact rods, resuspended in:		
200 mM KCl/20 mM Tris-HCl (pH 7.4)	1	leaky
200 mM KCl/20 mM Tris-HCl (pH 7.4) after 24 h at 4°C	67	leaky
"Non-depleted Tris rods" * in		
160 mM Tris-HCl, pH 7.4	21	leaky
160 mM Tris-HCl, pH 7.4 after 24 h at 4°C	100	leaky

* These preparations are defined under Materials and Methods and were used in a previous study [16].

The decrease of the calcium content of rods reflects a change of the calcium binding sites, which is analyzed by measuring the decay of the calcium binding capacity in different rod preparations in the presence of A23187 (Table V). With various external calcium concentrations up to five times the affinity of the binding sites, there is a proportional decay of calcium binding, suggesting a decrease in the capacity or a large shift of the affinity of the binding sites. These results explain the difference in response to A23187 of the intact rods as compared to the leaky Tris rods [16]. Whereas in the leaky rods addition of A23187, even in the absence of EGTA, gives a nearly complete release of calcium, no release is observed in intact rods under the same conditions. This difference cannot be explained by the presence or absence of an intact plasma membrane, since in the intact rods all calcium can be titrated stoichiometrically by EGTA in the presence of A23187 (Fig. 8A). In addition, the calcium binding capacity is largely retained in the stable leaky rods (not shown).

Effects of osmotic shock

Earlier observations [16] suggested that the loss of the stacked disk structure

TABLE V

DECAY OF CALCIUM BINDING IN DIFFERENT ROD PREPARATIONS

Calcium binding is determined in the presence of A23187 by Scatchard plots, similar as in Fig. 7.

Preparation	Half-time
Intact rods in sucrose-Ficoll medium, 0°C	>2 days
Intact rods in sucrose-Ficoll medium, 25°C	20 h
Intact rods, 50% of sucrose-Ficoll medium replaced by 200 mM KCl, 0°C	130 min
Intact rods, 50% of sucrose-Ficoll medium replaced by 200 mM KCl, 25°C	40 min
Intact rod pellet, resuspended in 200 mM KCl, 20 mM Tris-HCl (pH 7.4), 25°C	15 min

and the swelling of the disk are responsible for the decrease in stability described in the previous paragraph. To distinguish the immediate effects of disruption of the stacked disk structure from the subsequent ageing effects, the influence of osmotic shock on the Ca-Ca exchange have been investigated. Fig. 9 demonstrates that a shock with 3 vols. of water has almost no effect on Ca-Ca exchange. Under these circumstances the rod structure is preserved and the plasma membrane remains intact, according to the criteria used previously [19].

A shock with 6 vols. of water results in deformed rods, which are then visible as spherical objects under the light microscope. Similar observations with frog rods have been described by Hagins et al. [14]. After a shock with 10 vols. of water (rhodopsin concentration in the starting suspension should not exceed 15 μM), the turbidity of the suspension shows a steep decrease and no structure is visible any more under the light microscope. Most of the calcium is still present within individual disks (Fig. 2), but the rate of Ca-Ca exchange is greatly decreased (Fig. 9). The sensitivity to La^{3+} is not altered (Table IV). Net efflux of ^{45}Ca is still fairly slow (compare Fig. 2 with Table III), but the disks do not retain calcium upon addition of A23187, even in the absence of EGTA. This suggests that the binding sites for calcium are greatly altered.

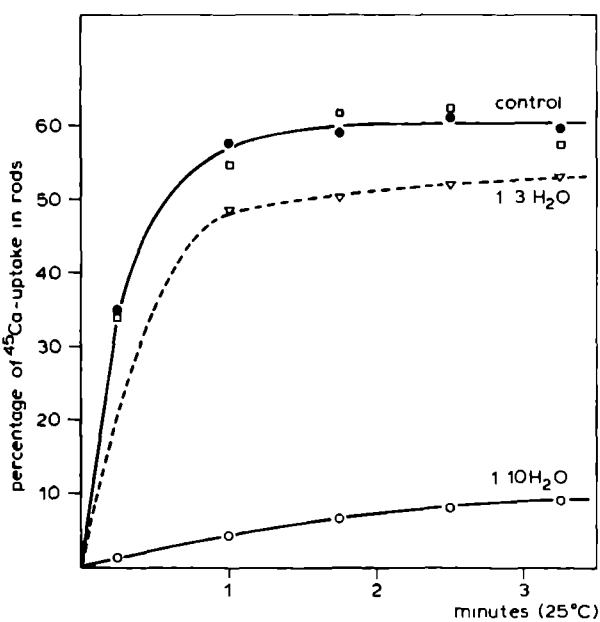

Fig. 9. Effect of osmotic shock on ^{45}Ca-equilibration. ^{45}Ca-uptake in rods is measured as a function of time at 25°C. Closed circles. Control, medium consists of 600 mM sucrose, 5% Ficoll 400, 20 mM Tris-HCl (pH 7.4). Open circles After previous lysis with 10 vols. of water, containing 5% w/v Ficoll 400. Final medium same as control medium. Triangles· After previous shock with 3 vols. of water. Final medium. same as control medium. Squares After a shock with 3 vols. of water and no change of medium. In the case of change of the medium (by centrifugation and resuspension) the data are related to a control sample, treated likewise.

Discussion

Calcium and rod outer segments

The intact isolated bovine rod outer segments (rods), used in this study and prepared according to the previous paper [19], contain 2—3 mol endogenous calcium per mol rhodopsin. Their higher calcium content, as compared to rods isolated in a calcium free medium (Table I), cannot be completely explained by uptake of calcium from the medium. Isolated rods do not accumulate appreciable amounts of calcium during a time interval (60 min) such as required for the isolation procedure. We rather presume that external calcium has a stabilizing effect on the rod structure, which results in better retention of endogenous calcium.

In the intact rods endogenous calcium appears to be mainly located within disks, since lysis in the presence of EGTA leaves the majority of the endogenous calcium in a compartment, which is not accessible to the EGTA in the washing medium. This is in agreement with the results of a histochemical localization technique [23]. The predominantly intradiskal location of endogenous calcium is consistent with the kinetic analysis of ^{45}Ca equilibration by Ca-Ca exchange, as shown in Figs. 4 and 6. The linearity of these plots indicates that all endogenous calcium pools exchange with the same rate constant. Although intact rods are generally believed to enclose two compartments, the cytosol and the intradiskal space, no indications for two consecutive reactions have been observed in our studies. In addition, ^{45}Ca equilibration in intact rods occurs with at least the same rate as in leaky rods. Thus we find no indication for the operation of two permeability barriers in these preparations. One interpretation of these results is that only a single barrier exists between intradiskal and external calcium. Alternatively, one must assume that in intact rods exchange across the disk membrane is much faster than observed in the various leaky or lysed rod preparations.

The endogenous calcium content of both intact rods (Fig. 3) and leaky rods [16] is essentially independent of the external calcium concentration. The predominant transport mode under the conditions used in this paper is therefore Ca-Ca exchange. This exchange is about three orders of magnitude faster than the net calcium transport. The half-time of net calcium efflux from rods (some hours) is comparable to that of the efflux of ^{45}Ca from liposomes, prepared from rod phospholipids [24]. Therefore, net calcium transport may reflect simple diffusion through the membrane bilayer down the calcium gradient, resulting from either a high or a very low external calcium concentration. Net calcium transport may als be governed by the transport rate (through diffusion) of the cotransported anion. The rod translocation system appears, under our conditions, restricted to exchange diffusion with a high unidirectional flux of 3.9 mol Ca^{2+}/mol rhodopsin per min. With the average rod dimensions of 1×20 μm and an overall rhodopsin concentration of 3 mM in the rod, a flux through the plasma membrane of 4.9 pmol Ca^{2+}/cm^2 per s or $2 \cdot 10^6$ Ca^{2+}/s per rod can be calculated.

The phenomenon of exchange diffusion and the presence of an external transport site, which exhibits saturation behaviour (Fig. 5), suggest the presence of a mobile carrier type translocation system [25]. The low Q_{10} value of

1.08 seems to plead against such a mechanism and is generally associated with a pore of channel type of mechanism [26]. Therefore, a neutral terminilogy (translocation system) is used, since a detailed molecular picture of the underlying system is not yet available.

The endogenous calcium content of intact rods, which is predominantly located within the disks, becomes dependent on the external calcium concentration upon addition of the ionophore A23187 (Fig. 7A). In isolated intact rods A23187 makes both the plasma and the disk membranes permeable to calcium (Fig. 8). The equilibrium between endogenous calcium and the external calcium concentration is then described by a linear Scatchard plot (Fig. 7b), which indicates the existence of a set of identical intradiskal binding sites with a maximal capacity of 8—9 mol calcium/mol rhodopsin and an affinity of 55 μM to calcium. A minor contribution of binding sites exposed to the cytosol cannot be excluded on the basis of the present data. The complete and reversible removal of endogenous calcium by EGTA is consistent with the notion that equilibration of free calcium concentrations between all compartments occurs in the presence of A23187. In view of the fact that A23187 exchanges calcium for protons, calcium can apparently be reversibly replaced by protons at the binding sites. Therefore, deviations of linearity in the Scatchard plot may be explained by the lack of sufficient exchangeable protons. From the binding curve and the endogenous calcium content of rods an apparent free intradiskal calcium concentration of 15—25 μM can be estimated for isolated intact rods without A23187. This means that more than 99% of the intradiskal calcium in rods must exist in a bound state.

Energetic considerations

Addition of A23187 results in a net efflux of calcium only when the external calcium concentration is below 15—25 μM. Complete efflux occurs when a strong chelator like EGTA is present externally (Fig. 8). The calcium content of rods is not affected by treatment with high concentrations of A23187 for several hours at 0° C, unless EGTA is added. This reinforces the conclusion that intact rods bind calcium, rather than sequester it by an energy requiring process. This is further strengthened by our observation that rods completely depleted of calcium in the presence of A23187 and EGTA, spontaneously rebind calcium to the original equilibrium levels when the external free calcium concentration is restored.

Furthermore, we observed that the calcium storage and the Ca-Ca exchange in rods are both preserved during storage of the rods at 4° C for up to 2 days. Under these conditions Ca-Ca exchange still persists (low Q_{10}) and no substantial amount of endogenous calcium is lost. On the other hand, the endogenous metabolic energy supply as measured by the ability to generate NADPH for the reduction of all-*trans* retinal following rhodopsin photolysis, is hardly affected by storage for two days at 4° C [19]. The following calculation shows that it is unlikely that hydrolysis of ATP is required for Ca-Ca exchange. From the unidirectional flux of 3.9 mol Ca^{2+}/mol rhodopsin per min, the Q_{10} of 1.08, a rhodopsin concentration of 3 mM and the assumption that approx. 1 mM ATP is present and is completely hydrolyzed in two days, it would follow that 29 000 mol Ca^{2+} would have been transported per mol ATP hydrolyzed. If,

alternatively, 2 mol Ca^{2+} would be transported per mol ATP hydrolyzed (the ratio found for the (Ca^{2+} + Mg^{2+})-ATPase system in sarcoplasmic reticulum and erythrocytes), the ATP supply would be exhausted in less than a minute. Neither is calcium storage sustained by an ATP-hydrolyzing system, since it is not affected by prolonged exposure (some hours at 0°C) to the combination of A23187 and a high (or intermediate) calcium concentration, which should maximally stimulate energy-requiring Ca-sequestering mechanisms. On the contrary, a substantial net uptake of calcium can be obtained in the presence of A23187 after previous depletion of calcium by EGTA (Fig. 7 and its discussion). Therefore, ATP is not required for net calcium uptake under these conditions. In addition, the ATP-effect on ^{45}Ca uptake, previously described, does not have the properties of the (Ca^{2+} + Mg^{2+})-activated ATPase pump system [16]. Finally, the decay of ATP has been reported to be fairly slow in frog rods under more physiological conditions [27,28]. In conclusion, none of the data presented in this paper indicate a significant involvement of metabolic energy in either the calcium translocation or the storage system. In agreement herewith purified rod membranes contain neither (Ca^{2+} + Mg^{2+})-ATPase [29, 30] nor (Na^+ + K^+)-ATPase [29,31].

From the present data the suggestion arises that in the rod outer segment ion fluxes occur primarily passively. The energy input to maintain asymmetrical ion fluxes could be supplied by the Na^+-K^+ pump, which is most likely located in the rod inner segment membrane. If the Na^+-K^+ pump is physically removed during isolation of the outer segments, it can be expected that new ionic equilibria are established within a few minutes, as suggested by rapid Ca-Ca exchange and Na-Ca exchange processes decribed in this paper and in Ref. 16. In support of this, the sodium dark current in the retina disappears with a half-time of 30 s, when the Na-K pump is specifically inhibited by ouabain [32]. These considerations supply the rationale for our use of a Na^+-free medium in isolating and maintaining the rod outer segments. In addition they indicate why experiments with these rod preparations should be restricted to short incubations after changing the external medium, since the exchange mechanisms might then rapidly establish a new equilibrium state. These points are illustrated for the intact retina by Sillman et al. [33], who have shown that after a 7 min exposure of the retina to ouabain in the absence of external Na^+, a receptor potential can still be recorded upon illumination, immediately after Na^+ is added back to the medium.

Comparison of different rod preparations

As discussed in the previous paper [19], it is essential for reproducible experiments with isolated rods to have a homogeneous preparation with well-defined properties. The various cattle rod preparations used in this paper illustrate how important this criterion is. Although the Ca translocation and storage systems are always present, characteristic parameters like rate constants and binding capacities vary considerably, and different responses to agents like La^{3+} (Table IV) and A23187 (Table V) are noted. Another critical point regards the stability of the preparation towards the medium conditions. For example, hypotonic shocks, sufficiently strong to abolish the stacked disk structure, appear to destroy the calcium translocation system and greatly affect the

calcium binding capacity, as indicated by Fig. 9 and by the calcium released in lysed rods upon addition of A23187. These observations are especially noteworthy since in most of the reports on a light-induced calcium release in isolated rods a hypotonically shocked or otherwise fragmented rod preparation has been used [6,7,10—13,17].

Therefore it seems essential to take into account the differences between the various preparations. In view of the consistent and reproducible experimental results obtained with stable intact and stable leaky rods, as defined in the previous paper [19], we prefer these preparations for studies of the calcium translocation and storage systems in rods. The sucrose-Ficoll medium does not seem to interfere with these systems. Substitutions of the major part of it by various electrolyte media has relatively minor effects at first (Table II), but after prolonged exposure, particularly after resuspension, large changes occur. This is illustrated by:

1. The half-time of the net Ca efflux, probably simple diffusion through the membrane bilayer, decreases from 165 min to 5 min (Table III).
2. The unidirectional Ca flux of the Ca-Ca exchange is greatly decreased (by lysis or in Tris rods).
3. The sensitivity of Ca-Ca exchange to La^{3+} is gradually abolished (Table IV).
4. The properties of the intradiskal calcium binding sites are drastically modified (Table V).

These changes must reflect modification of the membrane structure, probably by charge rearrangement, since they involve interactions with ions.

Concluding remarks

Intact cattle rods contain a calcium translocation system which can maintain high ion fluxes. This system bears some resemblance to the calcium exchange system described for nerve axon membrane [34,35], which will be discussed in a forthcoming communication on the ion selectivity of the rod translocation system.

The disks contain a large calcium binding capacity in equilibrium with free calcium concentrations in the micromolar range. Communication between external and intradiskal calcium occurs very rapidly by exchange. This conclusion is not easily reconciled with the calcium transmitter hypothesis of visual excitation [1]. Although the present data do not provide information on the calcium concentration of the rod cytosol, a crucial element in this hypothesis, there is no report that the disk membranes contain mechanisms to reduce the extradiskal, free calcium concentration to the required values of 1 μM or lower (cf. Ref. 36). Furthermore, in 'stable' rods, a fast light-induced shift of the intradiskal equilibrium of calcium binding appears to occur without detectable calcium release from disks [37].

The physiological relevance of the Ca-Ca exchange system in combination with the calcium storage capacity, described in this paper, remains to be established. In addition, we have to be aware of the limitations of isolated cell organelles in an artificial medium, when we wish to extend the conclusions from such preparations to the in vivo situation. When attempting to relate our observations to present views on the mechanism of visual excitation, these reservations should be kept in mind.

Acknowledgements

My thanks are due to Drs. F.J.M. Daemen, W.J. de Grip, J.J.H.H.M. de Pont, S.L. Bonting, P.J.G.M. van Breugel, L.H. Pinto and G.W.F. Borst Pauwels for critical discussions during the course of the work and during the preparation of the manuscript. The expert technical assistance and many helpful suggestions of Mr. A.A. Klompmakers and Mr. P.A.A Jansen are gratefully acknowledged. This investigation is supported in part by the Netherlands Organization for the Advancement of Basic Research (Z.W.O.), through the Foundation for Chemical Research in The Netherlands (S.O.N).

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Reprinted from

Biochimica et Biophysica Acta, 468 (1977) 259—270

BBA 77750

BIOCHEMICAL ASPECTS OF THE VISUAL PROCESS

XXXVI. CALCIUM ACCUMULATION IN CATTLE ROD OUTER SEGMENTS: EVIDENCE FOR A CALCIUM-SODIUM EXCHANGE CARRIER IN THE ROD SAC MEMBRANE

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(Received January 25th, 1977)

Summary

Accumulation of calcium has been studied in bovine rod outer segments (rods), isolated by sucrose density gradient centrifugation. Calcium-depleted rods are obtained by having ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetra-acetic acid (EGTA) present during isolation.

Rods thus isolated have a leaky plasma membrane, as shown by the effects of ionophore A23187 and by their light-induced phosphorylation behaviour. The accumulation of ^{45}Ca, determined by incubation followed by a single fast washing-filtration procedure, thus represents translocation across the rod sac membrane.

Accumulation in non-depleted rods is independent of the external calcium level and of ATP, suggesting exchange of ^{40}Ca by ^{45}Ca. In depleted rods in the presence of ATP there is net uptake, sigmoidally increasing with the external calcium concentration to the level attained in non-depleted rods. This net uptake is abolished by omission of ATP, its replacement by β,γ-methylene ATP and lowering the temperature to 0° C, suggesting involvement of enzymatic hydrolysis of ATP.

Replacement of KCl by NaCl in the medium causes marked inhibition of ^{45}Ca uptake, both net uptake and exchange. Oligomycin, ruthenium red, lanthanum and ouabain do not inhibit accumulation.

Efflux of ^{45}Ca from pre-loaded rods is slow in a KCl medium ($t_{1/2} \sim 30$ min at 25° C), but is greatly accelerated by addition of NaCl or Ca^{2+} ($t_{1/2} \sim 10$ s at 25° C).

It is concluded that the rod sac membrane contains a carrier system, which is sensitive towards Ca^{2+} and Na^{+} and which requires ATP for net uptake of Ca^{2+} but not for exchange transport of Ca^{2+} with Ca^{2+} or Na^{+}.

Abbreviation. EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetraacetic acid

Introduction

It has been suggested that Ca^{2+} acts as a transmitter in rod outer segments (rods) between the rod sac membrane and the rod plasma membrane [1]. The rod sacs would store Ca^{2+} Part of this would be released during photolysis of rhodopsin, thus increasing the cytoplasmic calcium concentration. The released Ca^{2+} would diffuse to the plasma membrane, where it would close sodium channels, thus reducing the sodium permeability of this membrane.

In our laboratory Hendriks et al [2] have found that frog rods, after careful and fast isolation in the presence of ATP, have a very high calcium content. They have also reported a shift in calcium distribution in these rods upon illumination after osmotic lysis. In cattle rods, which require a much more extensive and drastic isolation procedure, they find a rather variable and lower calcium content.

The high calcium content of rods, the probably saccular location and the fast release of Ca^{2+} from the rod sacs upon photolysis of rhodopsin would require the existence in the rod sac membrane of an efficient system for binding or translocation of calcium

Binding of calcium to rod sac membranes has been studied in our laboratory by means of the equilibrium dialysis method [3]. Cattle rods have been used in these and all further experiments. The binding studies indicate that at the presumed low cytoplasmic calcium concentration (approx. 10^{-6} M, see ref. 4) far too little calcium would be bound to the cytoplasmic side of the rod sac membrane to explain the high calcium content and the light-induced release of calcium.

Thus a calcium translocation mechanism must be considered Therefore, accumulation and efflux of calcium in cattle rods has been studied by means of ^{45}Ca. Through the use of calcium-depleted as well as non-depleted rods it has been possible to distinguish between net calcium uptake and exchange of ^{45}Ca with endogenous ^{40}Ca. These experiments have demonstrated the presence of a calcium translocation system in the rod sac membrane.

Methods and Materials

Isolation of cattle rod outer segments All procedures, except for the calcium determinations, are carried out in dim red light or darkness.

Cattle rods are normally isolated according to de Grip et al. [5], involving mild homogenization in Tris · HCl buffer (160 mM, pH 7.4), followed by sucrose density gradient centrifugation. After gradient centrifugation the rod layer is collected, diluted with three volumes of the ice-cold isolation medium, centrifuged (1000 × *g*, 15 min, 4° C) and resuspended in the desired medium. The preparation is stored in ice and used within 2 h. Calcium-depleted rods are prepared by addition cf 1 mM EGTA, an efficient calcium chelator, to the isolation medium.

Calcium determination. A 0.5 ml aliquot of rod suspension is incubated for 15 min at 18—20° C with 4 μM A23187 (gift of E. Lilly and Co., Indianapolis, Ind., U.S.A.) and 5 mM EDTA. The ionophore A23187 is added as an ethanolic

solution (final concentration ethanol <0.4%) with agitation. The suspension is centrifuged and calcium is determined in an aliquot of the clear supernatant. The calcium content of the sediment remaining after ionophore treatment is less than 1% of the original content (Drenthe et al., to be published).

Calcium is determined with a Pye Unicam SP1950 double-beam atomic absorption spectrophotometer. All samples contain 0.5% $LaCl_3$ to overcome anionic interference. Calcium standard solutions are prepared from anhydrous $CaCO_3$, which is dissolved in HCl containing 0.5% $LaCl_3$

Rhodopsin determination Rhodopsin is determined in a rod preparation by determining the 500 nm absorbance before and after complete bleaching in the presence of 1% Triton X-100 and 50 mM hydroxylamine The molar concentration is calculated by using a molar absorbance of 40 600 at 500 nm for rhodopsin [6].

Determination of ^{45}Ca accumulation 1 ml of cattle rod suspension (15—30 nmol rhodopsin) is incubated at 25° C in a medium containing 100 mM KCl, 20 mM Tris · HCl (pH 7.4), 2 mM $MgCl_2$, about 2 μCi ^{45}Ca (The Radiochemical Centre, Amersham, U.K., 1 Ci/50 mg Ca^{2+}), varying concentrations of $^{40}CaCl_2$ and other additions. Aliquots of 200 μl are transferred to 2.5 ml ice-cold washing medium on a borosilicate glass fiber filter (high flow rate, no adsorption of calcium), immediately followed by addition of another 2.5 ml ice-cold washing medium and suction through the filter. The entire washing procedure takes about 5 s. The washing medium has the same composition as the incubation medium, except for omission of ^{40}Ca and ^{45}Ca and addition of sufficient EGTA (0.25 mM) to chelate all calcium present. Blanks consist of ^{45}Ca-free suspension and radioactive isotope solution mixed in the washing medium and immediately subjected to the same washing and filtration procedure These blanks give the same values as tissue-free blanks. The filters are placed in counting vials filled with 10 ml Aquasol (New England Nuclear, Boston, Mass., U S A). Total radioactivity is determined by pipetting an aliquot of the labeled suspension directly into a counting vial, to which an unused filter is added.

The single fast washing-filtration procedure yields a rapid and complete separation between ^{45}Ca in the medium and ^{45}Ca accumulated inside the rods due to complete and instantaneous chelation by EGTA of all accessible ^{45}Ca.

Phosphorylation procedure 1 ml outer segment suspension (20—30 nmol rhodopsin) is preincubated at 25° C. Phosphorylation is started by addition of ATP (final concentration 1 mM) and 2 μCi [γ-^{32}P] ATP (The Radiochemical Centre, Amersham, U.K., 3 Ci/mmol). The sampling, washing and counting procedure is the same as used in the determination of ^{45}Ca accumulation except for the following modifications. The washing medium consists of ice-cold 0.12 M sodium phosphate buffer (pH 7.4) and two additional portions of washing medium are used. The filter is not allowed to run dry.

Results

Endogenous calcium content of isolated cattle rod outer segments

The calcium contents of non-depleted and depleted rods are 2.0 and 0.43 mol Ca^{2+}/mol rhodopsin, respectively (Table I). These endogenous calcium

TABLE I

ENDOGENOUS CALCIUM CONTENT OF VARIOUS CATTLE ROD OUTER SEGMENT PREPARATIONS

	Non-depleted	Depleted
Calcium content (mol Ca^{2+}/mol rhodopsin)	2.0	0 43
S.E.	0.4	0.09
Range	0.7—4.0	0 18—0.66
Number of preparations	8	5

contents must be taken into account in interpreting the results of ^{45}Ca accumulation experiments.

The rather variable results indicate that the rods easily lose calcium during the lengthy (2—2.5 h) isolation procedure. This point is further illustrated by the dramatic decrease of the calcium content when EGTA is present during isolation (depleted rods). Major contamination by extracellular calcium seems unlikely, since non-depleted rods in vitro do not show a net uptake of extracellular calcium (see Fig. 2 and its discussion).

^{45}Calcium accumulation as a function of time

Fig. 1 shows that ^{45}Ca accumulation in the presence of excess EGTA does not significantly exceed the isotope blank level. Accumulation in the absence of EGTA extrapolates at zero time to the isotope blank level, indicating the validity of the blank corrections. The rapid approach to a maximal level, many times higher than the blank level, indicates that ^{45}Ca is indeed accumulated inside rod outer segments.

Steady-state filling, i.e. the condition where influx of ^{45}Ca equals its efflux,

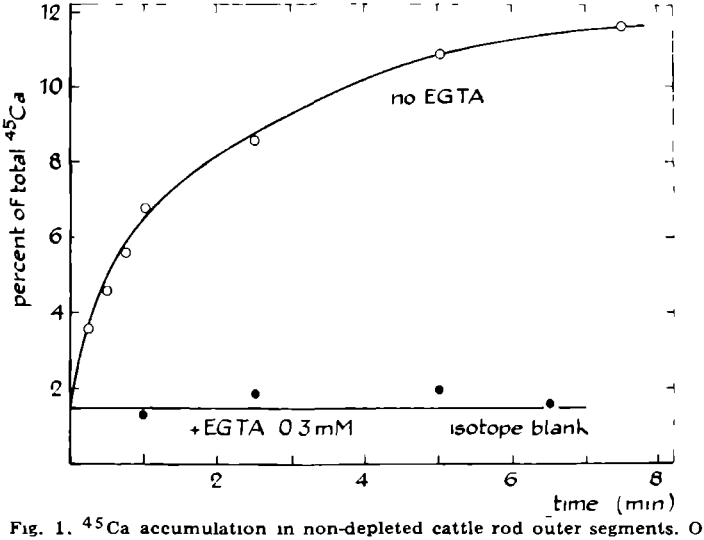

Fig. 1. ^{45}Ca accumulation in non-depleted cattle rod outer segments. Open symbols, no EGTA, closed symbols, 0.3 mM EGTA. The horizontal line represents the isotope blank level. Medium 100 mM KCl, 2 mM $MgCl_2$, 19 μM $CaCl_2$, ImM Tris · ATP, 20 mM Tris · HCl (pH 7.4)

is reached after 10—30 min incubation at 25° C. This is shown for non-depleted rods in Fig. 1, but it is also true for depleted rods. In all further experiments, where only the steady-state filling level is determined, a standard incubation time of 45 min at 25° C has therefore been used. Steady-state filling levels are calculated from triplicate determinations of the tracer content. In calculating the amount of accumulated calcium, the isotope is assumed to be equally distributed over endogenous and exogenous calcium in the steady-state. This assumption is supported by the horizontal curve in Fig. 2 for the calcium content of non-depleted rods, calculated from the accumulated ^{45}Ca.

Effect of medium composition on accumulation

In a number of experiments with depleted rods the major osmotic component in the accumulation medium has been varied (Table II). The only significant deviation is a large decrease in accumulation in the presence of 100 mM Na^+. Experiments at varying Na^+ concentrations indicate that 100 mM Na^+ has approximately maximal effect and that half-maximal effect is obtained somewhere in the range of 10—30 mM Na^+. We shall return to this observation in the section describing efflux experiments. Omission of ATP from the media gives lower, but relatively similar accumulation levels (not shown). In view of these results, the experiments reported in the next four sections have been carried out in the basic medium with 100 mM KCl present as the major osmotic component.

Effect of external calcium concentration

Fig. 2 combines the results from four experiments, in which depleted and

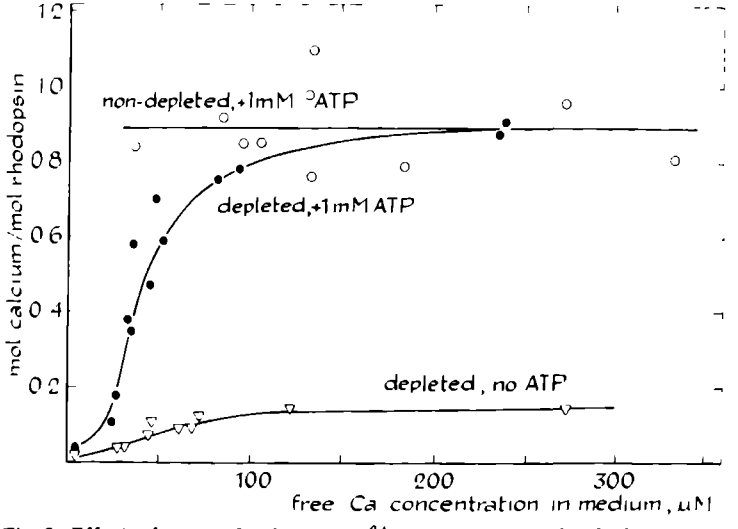

Fig. 2. Effect of external calcium on ^{45}Ca accumulation by depleted and non-depleted rods. Medium: 100 mM KCl, 2 mM $MgCl_2$, 20 mM Tris · HCl (pH 7.4). Free external calcium concentrations are calculated by subtracting from the total amount of calcium present, endogenous and exogenous, the amount of calcium incorporated in the rods. The results of four experiments are combined. ○, non-depleted rods, 1 mM ATP present in medium; ●, depleted rods, 1 mM ATP present in medium; ▽, depleted rods, no ATP present. Steady-state levels after 45 min incubation at 25° C are presented.

TABLE II

EFFECT OF MEDIUM COMPOSITION ON ^{45}Ca ACCUMULATION OF DEPLETED RODS

Basic medium contains $MgCl_2$, 2 mM; Tris HCl, 20 mM, ATP, 1 mM, $CaCl_2$, 50 μM (pH 7.4). Results refer to steady-state levels after 45 min incubation at 25° C and of the accumulation in the medium containing 100 mM KCl, with S.E. and number of experiments. Each experiment is carried out with a single depleted rod preparation.

Addition to basic medium	Accumulation	n
100 mM KCl	≡100%	7
100 mM NaCl	30 ± 6	7
100 mM Tris · HCl (pH = 7.4)	73 ± 9	2
100 mM sucrose	90 ± 2	3
200 mM sucrose	117 ± 22	4

non-depleted rods with and without ATP are compared. Non-depleted rods in the presence of ATP accumulate calcium to a level, which is independent of the external calcium concentration over a wide range (25—350 μM). The same effect is observed when ATP is omitted from the medium, except that the ^{45}Ca level remains about 35% lower (see Table III). This suggests that there is in these cases no net accumulation, but only an exchange between exogenous ^{45}Ca and endogenous ^{40}Ca.

Depleted rods show only in the presence of ATP at increasing calcium concentration a net calcium accumulation to a level equal to that of the original calcium content of the non-depleted rods. This suggests that the same calcium compartment is being filled with ^{45}Ca in the two cases. These experiments indicate that only after prior calcium depletion ^{45}Ca uptake experiments will yield true net uptake of calcium.

Effect of ATP on accumulation

ATP stimulates calcium accumulation significantly, but much more so in depleted than in non-depleted rods. The uptake in depleted rods is increased 429% and in non-depleted rods from the same batches of eyes by only 54% (Table III). Replacement of ATP by β,γ-methylene ATP (which is not hydrolyzed by ATPases and kinases) abolishes the stimulation of accumulation.

TABLE III

EFFECT OF ATP AND β,γ-METHYLENE ATP ON ^{45}Ca ACCUMULATION

Medium composition. 100 mM KCl, 2 mM $MgCl_2$, 50 μM $CaCl_2$, 20 mM Tris · HCl (pH 7.4) Steady-state levels after 45 min incubation at 25° C are presented as percent of the levels achieved in the absence of ATP. Results are averages with S.E. and number of experiments for depleted and non-depleted rods prepared from the same batch of eyes.

	No ATP	ATP (1 mM)		β,γ-Methylene ATP (1 mM)	
	%	%	n	%	n
Depleted rods	≡100	529 ± 50	4	69 ± 2	2
Non-depleted rods	≡100	154 ± 3	3	90 ± 2	2

This suggests that the stimulating effect of ATP involves its enzymatic hydrolysis.

Effects of temperature and ionophore on accumulation

In view of the stimulating effect of ATP on net calcium accumulation in depleted rods we have investigated the effects of the temperature. Table IV shows that low temperature only slightly lowers the accumulation in the absence of ATP, but completely abolishes the stimulation of ^{45}Ca uptake by ATP. This means that ^{45}Ca-^{40}Ca exchange shows little temperature dependence, whereas net uptake of calcium is greatly temperature dependent.

The presence of A23187 in the medium completely inhibits accumulation of calcium (Table IV, line 3). Conversely, addition of A23187 to a rod suspension after accumulation rapidly abolishes the calcium gradient (Fig. 3). This effect is virtually completely reversed by addition of 50 μM lanthanum, probably due to its complexation of the ionophore. These effects of A23187, which are consistently found in a large number (n = 18) of experiments, strongly suggest that accumulation involves translocation into the rod sacs, resulting in a concentration gradient across the rod sac membrane. Calculation shows that at low external calcium levels rod/medium ratios far above 1 are reached, e.g. a ratio of 25 at 50 μM external Ca^{2+}.

Effect of various substances on net accumulation

Table V shows the effects of some substances, which might influence ATP-dependent calcium accumulation in depleted rods. None of these substances has a clear effect, although ruthenium red (100 μM) may cause up to 60% inhibition in experiments with a high stimulation (3—5-fold) of accumulation by ATP.

Structural integrity of isolated rods

The calcium level in non-depleted rods and net calcium accumulation in depleted rods are very sensitive to mechanical disruption and ageing. Additional centrifugation for 15 min at 3000 × g causes 60 and 40% reduction, respectively, of calcium level and accumulation capacity, ageing by storage for 24 h at 4° C and freeze-thawing reduce both parameters by 70%, and lyophilization reduce them by virtually 100%. Electron microscopic inspection reveals that ageing and freeze-thawing result in loss of stacked-sac structure and swelling of

TABLE IV

EFFECT OF TEMPERATURE AND IONOPHORE A23187 ON ^{45}Ca ACCUMULATION OF DEPLETED RODS

Medium composition as in Table III. Results for one representative experiment from a total of four experiments. Steady-state levels after 45 min incubation at 25° C are presented as percent of the control level at 25° C.

Conditions	25° C	0° C
1. control (45 min at indicated temperature)	≡100%	53%
2. as 1, +1 mM ATP	403	64
3. as 2, +10 μM A23187	36	—

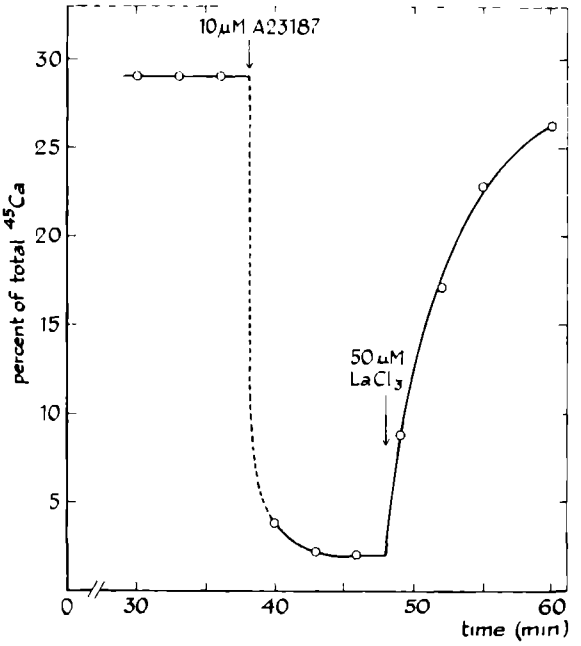

Fig. 3. Effect of ionophore A23187 on ^{45}Ca level of pre-loaded rods. Medium 100 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, 10 μM Ca^{2+}, 20 mM Tris · HCl (pH 7.4). Arrows indicate time of addition of A23187 and $LaCl_3$ to the indicated final concentrations.

the sacs, but does not permit a conclusion as to the intactness of the plasma membrane in these preparations.

We have used light-dependent phosphorylation of rhodopsin [7,8], which is nearly entirely located in the rod sac membrane, to establish whether externally applied solutes, in this case [γ-^{32}P] ATP, are immediately available to the rod sacs. Fig. 4 shows that the isolated rods are phosphorylated at exactly the same rate with or without prior freeze-thawing, indicating that the plasma membrane in the isolated rods does not function as a permeability barrier for small solutes.

Efflux of ^{45}Ca from pre-loaded rods

The calcium uptake experiments have been complemented by studies of ^{45}Ca

TABLE V

EFFECT OF VARIOUS SUBSTANCES ON ^{45}Ca ACCUMULATION OF DEPLETED RODS

Medium composition as in Table III. Steady-state levels after 45 min incubation at 25° C are presented as percent of the level achieved without added substance. Results are averages with S.E. for the indicated number of experiments (n).

Substance added	0 mM ATP	n	1 mM ATP	n
None	≡100%		≡100%	
Ouabain, 100 μM	80 ± 22	2	88 ± 7	4
Ruthenium red, 100 μM	100 ± 3	2	60 ± 21	3
Oligomycin, 5 μg/ml	92 ± 1	4	82 ± 6	4
La^{3+}, 50 μM	108 ± 3	3	122 ± 7	4

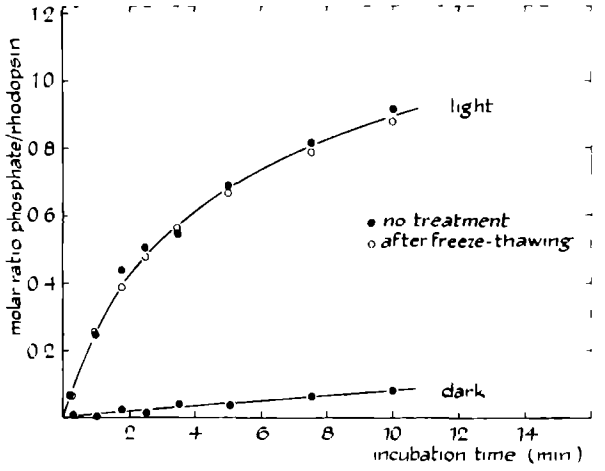

Fig. 4 Light-dependent phosphorylation of non-depleted rods. Rods, without (•) or after (○) prior freeze-thawing, are incubated at 25°C in a medium containing 100 mM KCl, 20 mM Tris HCl (pH 7.4) and 2 mM $MgCl_2$. Illumination with white light is started 30 s before addition of 1 mM Tris · ATP and 2 μCi [γ-^{32}P] ATP. Incorporation values for a control experiment in darkness are shown, and are deducted from the incorporation values in the light. Maximal incorporation is close to one phosphate per rhodopsin, and the incorporation curve approaches first-order kinetics, as would be expected when the ATP concentration and the kinase activity remain constant during the full incubation period.

efflux from rods pre-loaded in the absence of ATP. By omitting all calcium from the efflux medium and adding EGTA, net efflux of calcium can be measured in the absence of exchange fluxes, such as always occur in uptake experiments where even depleted rods still contain appreciable amounts of endogenous calcium (see Table I).

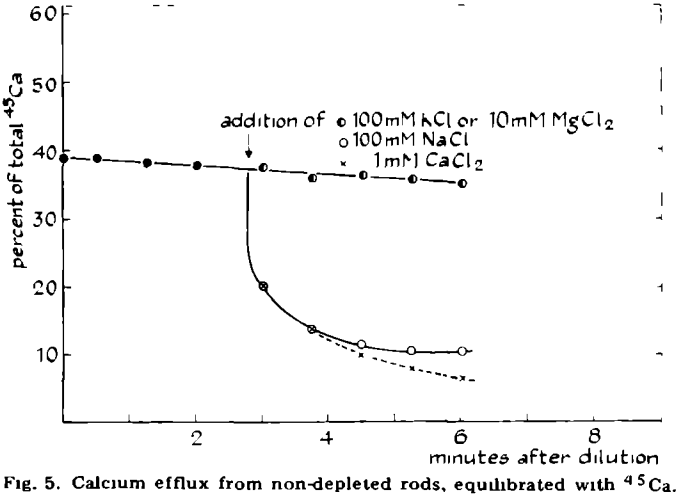

Fig. 5. Calcium efflux from non-depleted rods, equilibrated with ^{45}Ca. Equilibration medium: 100 mM KCl, 2 mM $MgCl_2$, 20 mM Tris HCl (pH 7.4), 2 μCi ^{45}Ca, 25°C. Efflux is started by 10-fold dilution (at 25°C) in the same medium without Ca^{2+} and 250 μM EGTA (•). The point on the ordinate represents a determination of tracer content before dilution. After 2.75 min a concentrated salt solution is added, which results in an additional final concentration of 100 mM KCl or 10 mM $MgCl_2$ (•) 100 mM NaCl (○) or 1 mM $CaCl_2$ (×).

The efflux experiment is started by the addition of nine volumes of a medium, lacking all calcium and containing 0.25 mM EGTA, to a suspension of rods preloaded with ^{45}Ca (Fig. 5). Efflux in a medium with 100 mM KCl (no Na^+) is slow ($t_{1/2} \sim 30$ min), which is also the case for efflux in media containing sucrose (100 or 200 mM) or Tris (120 mM) or lacking Mg^{2+}. This slow efflux indicates a low passive calcium permeability of the rod sac membrane.

However, when 100 mM NaCl is added to the medium there is a fast and large release of ^{45}Ca with a half-time of $t_{1/2} \sim 10$ s, whereas addition of 100 mM KCl or 10 mM $MgCl_2$ do not noticeably affect the efflux rate. This finding and the reduced ^{45}Ca accumulation in the presence of 100 mM NaCl (Table II) suggest the operation of a Ca^{2+}-Na^+ exchange carrier system. Addition of ATP to the efflux medium affects neither the slow nor the fast calcium efflux.

Addition of 1 mM ^{40}Ca to the efflux medium also greatly accelerates ^{45}Ca efflux (Fig. 5). The rapid phase of the efflux curve after addition of 1 mM $^{40}Ca^{2+}$ virtually coincides with that of 100 mM Na^+, suggesting that the same Ca^{2+}-Na^+ exchange carrier may operate here in ^{45}Ca-^{40}Ca exchange.

Discussion

Accumulation of ^{45}Ca by rods has previously been reported by several authors [9—14]. Although they use different terms, binding, uptake and accumulation, it may be assumed that in all these cases accumulation of ^{45}Ca inside rod outer segments or rod outer segment fragments has been measured, since all authors use sampling procedures based on dilution with isotope-free media. Unfortunately, rather little information about the accumulation system has been gained from these studies Neufeld et al. [10] and Mason et al. [11] have used sonicated rod material and Weller et al. [14] have applied a severe hypo-osmotic shock to their rod preparation, so that in these cases the original intact rod sac structure has presumably been lost. Hemminki [12] treats his earlier results as representing a mere binding process, neglecting the accumulation through translocation, which according to our results occurs even in the absence of ATP. His later attempt to distinguish between binding and active transport through the use of ATP and the ionophore A23187 [13] suffers from the fact that he makes the questionable assumption that the isolated bovine rods have an intact plasma membrane Moreover, in none of these studies has the endogenous calcium content been taken into account or removed, which at the low external calcium concentrations generally used may lead to false net uptake curves.

For the accumulation experiments in this paper we have used cattle rods isolated by means of sucrose density gradient centrifugation. The average calcium content, immediately after centrifugation, is 2.0 mol Ca^{2+}/mol rhodopsin for non-depleted rods, and 0.4 mol Ca^{2+}/mol rhodopsin in EGTA-depleted rods.

Parallel losses in calcium content and accumulation capacity are caused by centrifugation and resuspension, ageing, freeze-thawing and lyophilization. Conversely, depleted rods are capable of net uptake of calcium until the calcium content of non-depleted rods is reached. These observations suggest the presence of a single well-defined, structure-dependent calcium storage and accumulation capacity.

The rapid ^{45}Ca efflux from preloaded rods upon addition of A23187, Na^+ or $^{40}Ca^{2+}$ suggests that the rods behave like a two-compartment system. The phosphorylation experiment shows that the plasma membrane does not constitute a rate-limiting barrier to small solutes Hence the two compartments appear to be medium and intrasaccular space, and accumulation of calcium must involve translocation across the rod sac membrane The achievement of rod/medium ratios far above 1 and the rapid abolition of such a ratio by A23187 indicate that entering calcium can be translocated against a concentration gradient

In our accumulation and efflux experiments three modes of calcium translocation across the rod sac membrane can be distinguished. The first mode is the net uptake of calcium in depleted rods, which is clearly ATP dependent. The steady-state level is sigmoidally related to the external calcium and maximally reaches the originally present calcium level. Enzymatic hydrolysis of ATP appears to be involved, since β,γ-methylene ATP cannot replace ATP and cooling to 0° C abolishes the stimulating effect of ATP. The second mode of translocation is a Ca^{2+}-Ca^{2+} exchange process, which is observed in ^{45}Ca accumulation by non-depleted rods as well as in Ca^{2+}-stimulated ^{45}Ca efflux from preloaded rods. This process appears to be independent of ATP and has a low temperature coefficient ^{45}Ca accumulation by Ca^{2+}-Ca^{2+} exchange proceeds to a calcium level, which is virtually independent of the external calcium concentration in a range of 25—350 μM The third mode of translocation is a Ca^{2+}-Na^+ exchange process, which is indicated by the fact that external Na^+ inhibits calcium accumulation and stimulates calcium efflux The effects of external Ca^{2+} and Na^+ appear to be selective, since K^+ and Mg^{2+} do not stimulate Ca^{2+} efflux.

These three modes of translocation seem to be mediated by a single system, since (1) net uptake by depleted rods in the presence of ATP leads to about the same calcium level (determined with ^{45}Ca) as reached by exchange uptake in non-depleted rods, (2) net ^{45}Ca uptake and ^{45}Ca-^{40}Ca exchange uptake are similarly reduced by external Na^+, (3) conversely, external Na^+ accelerates ^{45}Ca efflux, and (4) the ^{45}Ca efflux rates after addition of external Na^+ or Ca^{2+} are about equal.

This translocation system differs from the calcium pumps in sarcoplasmic reticulum [15—17], mitochondria [15,18,19] and erythrocytes [15,20,21], as indicated by the ineffectiveness of lanthanum, oligomycin and ruthenium red and by the effects of external sodium on the steady-state filling levels of the rods. In accordance with this, we have so far not been able to detect a (Ca^{2+} + Mg^{2+})-activated ATPase activity in rod outer segment preparations [22]. How the system in the rod sac membrane utilizes the energy derived from hydrolysis of ATP in the case of net uptake is unclear. Coupling to a (Na^+ + K^+)-ATPase pump system, i.e. a calcium accumulation system driven by a sodium gradient, also seems unlikely, since ouabain does not affect calcium accumulation in rods

The translocation system, in so far as it seems to be capable of both Ca^{2+}-Na^+ and Ca^{2+}-Ca^{2+} exchange, seems to resemble the calcium extrusion systems operating in plasma membranes of nerve, muscle and other cells [15]. The latter systems are also selectively activated by Ca^{2+} and Na^+ and appear to utilize ATP under certain conditions [23,24], but apparently not through a (Ca^{2+} + Mg^{2+})-activated ATPase system as occurring in sarcoplasmic reticulum

Acknowledgements

Our thanks are due to Mr. A.A. Klompmakers for expert technical assistance and valuable suggestions. This investigation is supported in part by the Netherlands Organisation for the Advancement of Basic Research (Z.W.O.), through the Foundation for Chemical Research in the Netherlands (S.O.N.).

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ION SELECTIVITY OF THE CATION TRANSPORT SYSTEM OF ISOLATED INTACT CATTLE ROD OUTER SEGMENTS: EVIDENCE FOR A DIRECT COMMUNICATION BETWEEN THE ROD PLASMA MEMBRANE AND THE ROD DISK MEMBRANES

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Key words: Na^+-Ca^{2+} exchange, ion channels, membrane connections, vision (Rod outer segment)

SUMMARY

The ion selectivity of cation transport through the plasma membrane of isolated intact cattle rod outer segments (rods) is investigated by means of ^{45}Ca-exchange experiments and light-scattering experiments. These techniques appear to provide complementary information: the ^{45}Ca-experiments (^{45}Ca-fluxes in rods) describe electroneutral antiport, whereas the light-scattering experiments (shrinkage and swelling of rods upon hypertonic shocks with various electrolytes) reveal electrogenic uniport. Electroneutral symport of ions (salt transport) does not take place without addition of external ionophores and application of salts of weak acids.

1. Intact rods recover from a hypertonic shock in the presence of FCCP when lithium-, sodium- and potassium-acetate are applied, but not when ammonium chloride, calcium- and magnesium-acetate are used. This indicates that the plasma membrane of isolated intact cattle rods is relatively permeable to net transport of Na^+, Li^+ and K^+ ions, and relatively impermeable to net transport of Cl^-, Mg^{2+} and Ca^{2+} ions under conditions that no diffusion potentials arise.

2. Rapid ($t_{\frac{1}{2}} < 1$ min) efflux of ^{45}Ca from preloaded intact rods is observed when Na^+, Ca^{2+}, Sr^{2+} and, under certain conditions also, Ba^{2+} ions are added to the external medium.

Li^+, K^+, Rb^+, Cs^+, Mg^{2+} and Mn^{2+} ions are ineffective in this respect as well as protons at pH = 7.4. It is concluded that ^{45}Ca-efflux reflects electroneutral exchange diffusion of internal ^{45}Ca with external Na^+, Ca^{2+}, Sr^{2+} and Ba^{2+} ions respectively.

3. All tested cations lower the rate of ^{45}Ca-uptake. The latter can be described by a single rate constant indicating a homogeneous rod preparation and a homogeneous endogenous Ca^{2+} pool. However, only those cations, which stimulate ^{45}Ca-efflux from preloaded rods lower the final equilibrium of ^{45}Ca-uptake. Except for the effects of K^+, Rb^+ and Cs^+ ions the reduction of the rate of ^{45}Ca-uptake by external cations appears to arise from competition for a common site on the plasma membrane. The observed affinities for this site do not correlate with actual transport (as indicated by the ability to stimulate ^{45}Ca-efflux).

4. K^+ ions increase the affinity of the exchange diffusion system to Ca^{2+} ions from 1 µM to 0.16 µM and change the relative affinities with respect to Ca^{2+} for the other cations (Na^+, Mg^{2+}, Mn^{2+}, Sr^{2+}, Ba^{2+}). Furthermore, the maximal rate of Ba-Ca exchange is strongly stimulated by K^+ ions, whereas the maximal rate of Ca-Ca exchange is reduced at saturating Ca^{2+} concentrations.

5. The exchange diffusion transport mode can be turned off by external Na^+ ions in a process not of stochastic nature, which implies interdependence of individual transport entities and which results in an inhomogeneity of the endogenous Ca^{2+} pool. K^+ ions act as antagonists of Na^+ ions

in this effect.

The relevance of these findings is discussed in relation to the general accepted view, that a diffusable transmitter in the rod cytosol communicates the photochemical event in the disk membrane to the electrical properties of the plasma membrane. It is argued that the exchange diffusion system present in the plasma membrane of isolated cattle rods has a number of properties in common with the system responsible for the dark current through the outer segment of a rod cell in the retina. It is concluded that the exchange diffusion transport mode of the cation transport system in the plasma membrane of isolated cattle rods has access to both the extracellular side of the plasma membrane as well as the disk interior. Under these conditions it behaves as a single system, which exchanges cations directly from the extracellular space to the disk interior, whereas the disk membranes do not appear to contain a separate Ca^{2+} transport system.

INTRODUCTION

Experiments on the electrical behaviour of the vertebrate retina have suggested that Ca^{2+} ions play an important role in modulating the ion fluxes underlying the mechanism of visual excitation (1-6). The excitatory mechanism of rod photoreceptor cells is localized in the rod outer segment and therefore the Ca^{2+}-metabolism of these organelles has been investigated in this laboratory. For this purpose a new procedure was devised, which stabilizes the outer segment structure and allows purification of isolated cattle rod outer segments (rods) with either a functionally intact or leaky plasma membrane (7). Using these preparations, it appeared possible to preserve and characterize a Ca^{2+}-translocation system and a Ca^{2+}-binding capacity in a reproducible way (8).

It was found that the endogenous Ca^{2+}-pool of the intact cattle rods is primarily bound to intra-diskal binding sites and exchanges with external Ca^{2+} by the operation of a translocation system, which performs exchange diffusion without net transport (8).

In the present study the ion selectivity of this cation translocation system is investigated with a special emphasis on the interrelation between Ca^{2+} and Na^{+} ions. Because of the accuracy of the methods to assay and analyse ^{45}Ca-fluxes, data on the latter are found to be very useful also in the study of the effects of other ions. The cation translocation

system, described in this study, is shown to share a number of properties with the system responsible for the dark current of Na^+ ions in the vertebrate retina.

MATERIALS AND METHODS

Preparations and general procedures

All procedures with rod outer segments are carried out in darkness or in dim red light.

Stable cattle rod outer segments (rods) with either a leaky or an intact plasma membrane are prepared as described before (7). Whenever the term rods is used, it always refers to the stable intact rod preparation. Other rod preparations are explicitely defined in the text. The rod preparations are stored in the dark at $4^{o}C$ and as a concentrated suspension (100-200 µM rhodopsin) in the standard medium. The standard medium contains 600 mM sucrose, 5% w/v Ficoll 400 and 20 mM Tris-HCl (pH = 7.4). The sucrose-Ficoll solution is deionized by passing it over a mixed-bed ion exchange column before use.

Intact rods remain intact as determined by the criteria used before (7) during all manipulations imposed on them in this study (i.e. electrolyte additions, osmotic manipulation, addition of various ionophores).

The Ca^{2+}-content of the rod preparations is determined by the "ionophore extraction" method described before (8). The Ca^{2+} determinations are performed on a Pye Unicam SP 1950 double-beam atomic absorption spectrophotometer.

Rhodopsin determinations are performed according to the standard procedures of this laboratory (9).

^{45}Ca-experiments

All ^{45}Ca experiments are performed at $25^{o}C$ and use a final rhodopsin concentration of 15-25 µM in the standard

medium. A preincubation time of 10 minutes is used to thermally equilibrate the suspension from 4°C to 25°C. ^{45}Ca fluxes in rods are assayed by applying the rapid filtration technique described before (10) to samples withdrawn from the incubated suspension at the indicated times. All ^{45}Ca contents described in this study refer to this assay. Throughout all the experiments the washing medium contains 600 mM sucrose, 20 mM Tris-HCl (pH = 7.4) and 250 µM EGTA, which removes all adherent calcium (8,10). Radioactivity is counted in 10 ml Aquasol (new England Nuclear, Boston, USA) in a liquid scintillation counter.

The average <u>total</u> Ca^{2+} concentration in a rod suspension, containing 15-25 µM rhodopsin, amounts to 80-150 µM, of which 50-90 µM is <u>endogenous</u> Ca^{2+} leaving an <u>external</u> Ca^{2+} concentration of 30-60 µM. This means an average total Ca^{2+} content of purified cattle rods of 5.7 mol Ca/mol rhodopsin of which about 60% (3.4 mol Ca/mol rhodopsin) is endogenous Ca^{2+}, localized within intact rods (8). To this suspension of cattle rods the various tested cations (chloride salts) are added simultaneously with ^{45}Ca at the start of the incubation in the influx experiments, or are added after a prior 10 minute equilibration with ^{45}Ca at the start of the efflux experiments. In order to avoid aspecific effects due to the rather precarious (in)stability of the rods under various medium conditions (8) the ^{45}Ca-influx and -efflux experiments are restricted to at most 5 minutes. In view of the insensitivity of the rods to osmotic manipulation

electrolyte additions up to 120 mosmolar are not osmotically compensated for by leaving out sucrose. Deviations from the normal procedure are explicitely stated in the text.

Kinetic analysis of the ^{45}Ca-experiments

When no net Ca^{2+}-movements occur in rods ^{45}Ca-equilibration can be formally described by two opposing first order reactions. The resulting equations are:

$$\ln[X_f/(X_f-x)] = [k/X_f]t \quad \ldots\ldots\ldots \quad (1)$$

and

$$v = k(1-X_f)Ca_t \quad \ldots\ldots\ldots \quad (2)$$

X_f is the fraction of the total radioactivity in rods after equilibration, x is the fraction of the total radioactivity in the rods at time t, k is the rate constant, v is the unidirectional flux of Ca-Ca exchange, and Ca_t is the total Ca^{2+} concentration in the suspension. In all preparations the v_m (maximal velocity) is used as a reference and is defined as the observed velocity of the unidirectional ^{45}Ca-flux in a rod suspension without any additions, i.e. at an external Ca^{2+} concentration of 30-60 µM. This concentration is sufficient to saturate the transport system (8). Suspensions with low free external Ca^{2+} concentrations are obtained by addition of various amounts of EGTA, and the resulting free Ca^{2+} concentrations are calculated according to Caldwell (11).

Osmotic experiments

Certain aspects of the permeability properties of the plasma membrane of intact rods are investigated by an osmotic technique. A hypertonic shock on osmotically active

particles results in a shrinkage of the particles, which can be monitored by an increase of light-scattering at wavelenghts smaller than the dimensions of the particle. With permeable electrolytes the shrinkage is transient and a recovery is observed. The measurements are made in a Pye Unicam SP1750 spectrophotometer or a Beckman UV5260 spectrophotometer at a wavelength of 700 nm. The cuvette is placed directly in front of the photomultiplier. In order to obtain sufficiently large apparent absorbance changes the starting osmolarity of the rod suspension is reduced threefold immediately before use.

The rhodopsin concentration ranges from 5-15 μM and the experiments are performed at room temperature. The hypertonic shock is obtained by increasing the osmolarity of the suspension with 100 mosmolar by the addition of the various electrolytes. In order to induce a permeability for the various cations different ionophores are used. FCCP* (final concentration 5 μM) is used as an exclusive protonophore, Gramicidin D (final concentration 3 μM) performs electrogenic transport of protons and monovalent cations, and A23187 (gift of E. Lilly & Co., Indianapolis, USA; final concentration 5 μM) exchanges divalent cations and protons. The ionophores are added as an ethanolic solution (final ethanol concentration 0.5% v/v). Furthermore, the fact has been used, that NH_3 and acetic acid permeate rapidly through lipid bilayers.

With the procedure used mixing artifacts cannot be avoided. Therefore, the fast kinetics of recovery (< 1 min)

*FCCP = carbonylcyanide p-trifluoromethoxyphenylhydrazone

cannot be evaluated properly and the uncertainty in the amplitude amounts to about 10% of the initial apparent absorption change observed.

Results

Effects of various cations on ^{45}Ca-fluxes in isolated cattle rods

Addition of ^{45}Ca to a suspension of intact cattle rods results in a rapid ($t_{\frac{1}{2}}$=12 sec) uptake of ^{45}Ca in exchange with endogenous ^{40}Ca and leads to a complete equilibration of the external and endogenous Ca^{2+} pools (8). Stimulation of ^{45}Ca-efflux after previous ^{45}Ca-equilibration of the rods is a most sensitive test to establish which cations may replace Ca^{2+} ions in the exchange diffusion transport. Fig. 1

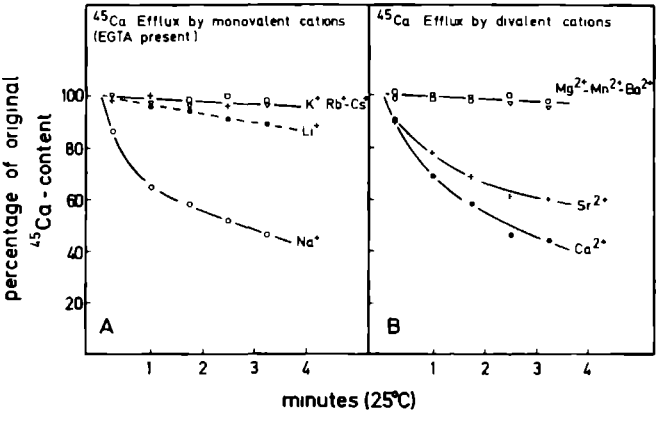

Fig. 1:

Efflux of ^{45}Ca from preloaded -rods in the standard medium (600 mM sucrose, 5% w/v Ficoll 400 and 20 mM tris-HCl at pH=7.4) at 25°C.

A In the presence of 250 µM external EGTA and after addition of the indicated chloride salts at the start of the incubation to a final concentration of 50 mM. LiCl (-•-); NaCl (-o-); KCl (-x-); RbCl (-▽-); CsCl (-□-).

B After addition at the start of the incubation to a final concentration of 1 mM: $MgCl_2$ (-▽-); $CaCl_2$ (-·-); $SrCl_2$ (-+-); $BaCl_2$(-□-); $MnCl_2$(-•-). The data are expressed relative to the ^{45}Ca-level after the previous equilibration. One hundred percent represents a Ca^{2+} content of 4.6 mol Ca^{2+}/mol rhodopsin.

demonstrates that Na^+, Ca^{2+} and Sr^{2+} ions do stimulate ^{45}Ca-efflux from pre-equilibrated rods, whereas the other tested ions (Li^+ ions may have a minor effect) do not evoke a significantly larger ^{45}Ca-efflux than the control. From the data in a previous study (8) it can be concluded that also La^{3+} ions and protons (the latter at the pH used and, in view of the very slow ^{45}Ca-efflux against external EGTA) are ineffective in this respect.

When ^{45}Ca is added to a rod suspension simultaneously with the tested cations ^{45}Ca-uptake is retarded in all cases (Figs. 2 and 3). This means that the tested cations decrease

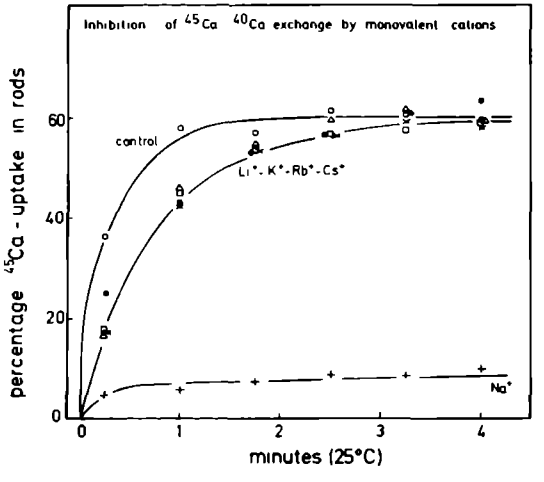

Fig. 2:

Effect of monovalent cations on ^{45}Ca-uptake in rods. In addition to the standard medium the indicated electrolytes are added at the start of the incubation at 25°C to a final concentration of 50 mM.
no additions (-o-); LiCl (-□-); NaCl (-+-); KCl (-Δ-); RbCl (-x-);CsCl (-•-).
The data are presented as the percent of total ^{45}Ca added. Total Ca^{2+} in the suspension amounted to 179 µM.

the rate of ^{45}Ca-^{40}Ca exchange. In accordance with Fig. 1

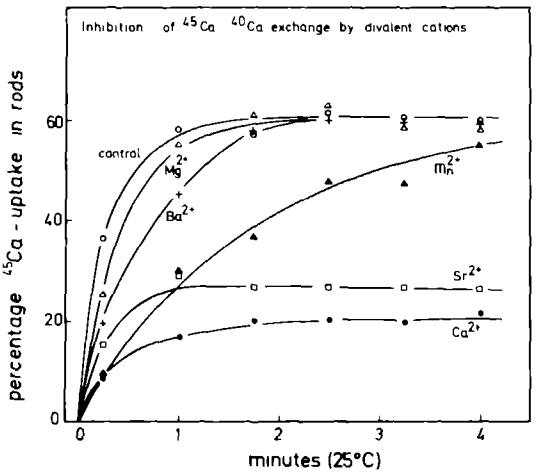

Fig. 3:
Effect of divalent cations on ^{45}Ca-uptake in rods. In addition to the standard medium the indicated electrolytes are added at the start of the incubation at 25°C to a final concentration of 250 µM.
no additions (-o-); $MgCl_2$ (-Δ-), $CaCl_2$ (-•-), $SrCl_2$ (-□-); $BaCl_2$ (-+-); $MnCl_2$(-▲-).
The data are presented as the percent of total ^{45}Ca added. Total Ca^{2+} in the suspension amounted to 179 µM.

the equilibrium level of ^{45}Ca-uptake is not affected by those cations, which do not stimulate ^{45}Ca-efflux. As expected from Fig. 1 Na^+, Ca^{2+} and Sr^{2+} ions do reduce the equilibrium level of ^{45}Ca-uptake. The ^{45}Ca-equilibrium levels reflect the ^{40}Ca-distribution (expressed in mol ^{40}Ca/mol rhodopsin). Therefore, when Ca^{2+} and Sr^{2+} ions are added, the ^{45}Ca-equilibrium levels observed in influx and efflux experiments such as shown in Fig. 1B resp. Fig. 3, differ by at most ten percent. Because of the presence of external EGTA a complete release of ^{45}Ca by Na^+ ions could be expected, but does not occur (Fig. 1). This is investigated in more detail in a later section.

Single ion effects: divalent cations

Considering the cause of the difference observed in Fig. 1B between the two classes of divalent cations, the possibility has to be investigated, whether the selectivity of the rod binding capacity (8) rather than the selectivity of the rod transport system determines this distinction. Fig. 4 shows that in the presence of the added exchange

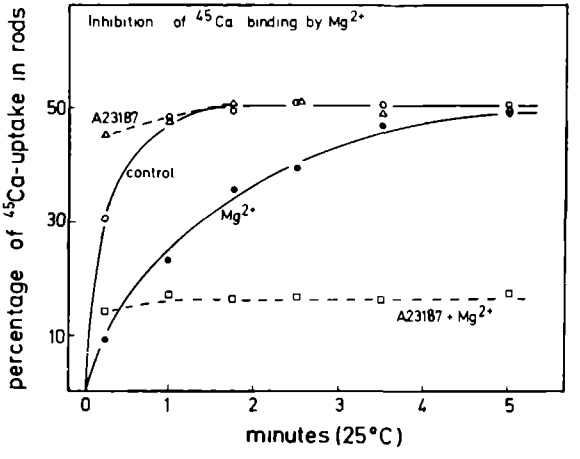

Fig. 4:
Effect of Mg^{2+} on ^{45}Ca-equilibration with and without the presence of A23187. In addition to the standard medium at 25°C:
no additions (-o-), 500 µM $MgCl_2$ (-•-); 5 µM A23187 (-Δ-); 5 µM A23187 + 500 µM $MgCl_2$ (-□-).
Mg^{2+} ions are added at the start of the incubation, A23187 is added at the start of the 10 minute preincubation as an ethanolic solution (final ethanol concentration 0.5% v/v).
The data are presented as the percent of total ^{45}Ca added. Total Ca^{2+} amounted to 37 µM, rhodopsin concentration was 12 µM. To obtain a clear picture the conditions were chosen so that ionophore addition did not change the final ^{45}Ca-equilibrium level.

carrier A23187 Mg^{2+} ions (and similarly Mn^{2+} and Ba^{2+} ions, not shown) do affect the equilibrium level of ^{45}Ca-uptake. The A23187 makes the binding sites, which store endogenous Ca^{2+} aspecifically accessible to external divalent cations. This experiment demonstrates that the properties of the rod

cation translocation system and not those of the binding capacity determine which divalent cation can promote ^{45}Ca-efflux, most likely by being transported itself into rods in exchange for internal ^{45}Ca. Furthermore this experiment illustrates that the ionophore A23187 resides in both the plasma membrane and in the disk membranes, because in leaky rods Mg^{2+} ions do not stimulate ^{45}Ca-efflux in the absence of A23187 (10).

Although Mg^{2+} ions (and similarly Mn^{2+} and Ba^{2+} ions) are apparently not transported by the exchange diffusion system, they do seem to compete with external Ca^{2+} ions for a common site. Higher Mg^{2+} concentrations give rise to a stronger reduction of the ^{45}Ca-^{40}Ca exchange rate. Fig. 5

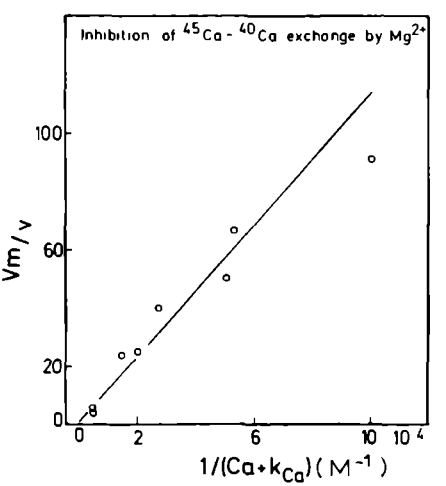

Fig. 5:
Analysis of the inhibition of the ^{45}Ca-^{40}Ca exchange rate by Mg^{2+} in the standard medium at 25°C. The data points are obtained from kinetic plots similar as shown in Figs. 9 and 10, and are plotted according to:

$$v_m/v = 1 + (Mg^{2} \times k_{Ca}/k_{Mg})/(Ca^{2+} + k_{Ca})$$

This equation describes competitive inhibition of Ca^{2+} transport by Mg^{2+}. It is obtained by dividing the Michaelis-Menten expression for Ca^{2+} transport in the absence of Mg^{2+} (v_m) by that in the presence of Mg^{2+} (v). Further same symbols as in Table I.

shows an analysis of the unidirectional ^{45}Ca-flux at an external Mg^{2+} concentration of 5 mM and as a function of the external Ca^{2+} concentration. A linear plot indicates competitive inhibition. Assuming competitive inhibition and using an affinity towards Ca^{2+} ions of 1 μM (8) the affinities (dissociation constants) of a number of tested divalent cations for the common site of the exchange diffusion system are calculated. The results are given in Table I and demonstrate that a high affinity (small dissociation constant) is not necessarily correlated to actual transport as indicated by the ability to stimulate ^{45}Ca-efflux (Fig. 1B).

TABLE I

The affinities of the exchange diffusion system for multivalent cations.

cation	k (μM) ± S.E. (number of observations)
Mg^{2+}	4.7 ± 0.6 (8)
$^{40}Ca^{2+}$	1.2 ± 0.2 (9)
Sr^{2+}	1.6 + 0.2 (3)
Ba^{2+}	3.5 ± 0.2 (2)
Mn^{2+}	0.9 + 0.2 (4)
La^{3+}	0.15 + 0.03 (9)

The affinities are obtained as inhibition constants of the ^{45}Ca-^{40}Ca exchange rate from plots as shown in Fig. 9 and are calculated according to the equation:

$$v/v_m = Ca^{2+}/[Ca^{2+} + k_{Ca}x(1 + Mi^{2+}/k_{Mi})],$$

where v is the observed rate of ^{45}Ca-^{40}Ca exchange, v_m is defined as the observed rate of ^{45}Ca-^{40}Ca exchange without additions to the rod suspension (see Materials and Methods), Ca^{2+} is the free Ca^{2+} concentration in the external medium, k_{CA} is the affinity (dissociation constant) of the transport system for Ca^{2+} ions and a value of 1 μM is used (8), k_{Mi} is the affinity for the inhibiting cation, and Mi^{2+} is the external concentration of the inhibiting cation. In the case of $^{40}Ca^{2+}$ as inhibiting cation extra added $^{40}Ca^{2+}$ competes with Ca^{2+} already present in the suspension and results in an affinity, which is expected and found to be identical with k_{CA}. The data of Table I refer to a rod suspension in the standard medium.

Single ion effects: monovalent cations

The observation in Fig 1A could suggest that Na^+ and possibly Li^+ ions compete with Ca^{2+} ions for the common external site. Qualitatively consistent with this suggestion is the observation that reduction of the external Ca^{2+} concentration (but still $\gg K_{Ca}$) in media, containing Li^+ ions resp. Na^+ ions, lowers the rate of ^{45}Ca-^{40}Ca exchange (Fig. 6)

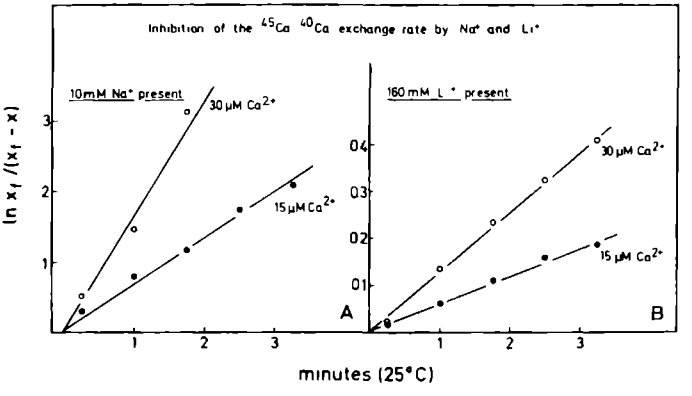

Fig. 6.

Competitive effects of Na^+ and Li^+ on the ^{45}Ca-^{40}Ca exchange rate. The data are plotted according to equation (1). Temperature 25°C.
A In addition to the standard medium 10 mM NaCl and external Ca^{2+} 30 µM (-o-); external Ca^{2+} reduced by addition of 15 µM EGTA (-•-). (see legend of Fig. 14 and the discussion of that figure)
B 160 mM LiCl replaces 480 mM sucrose and 4% w/v Ficoll 400 in the standard medium. Further conditions and symbols is in A.

in a similar way as observed previously for the divalent cations (Fig. 5).

A different case appears to be represented by K^+ ions, since it was previously found that a high concentration of K^+ ions does not reduce the (apparent) affinity of the exchange diffusion system for Ca^{2+} ions (Ref. 8, Fig. 5). In agreement with those observations K^+ ions exposed to the rod suspension simultaneously with ^{45}Ca do not show competitive

effects on the rate of ^{45}Ca-^{40}Ca exchange (Fig. 7).

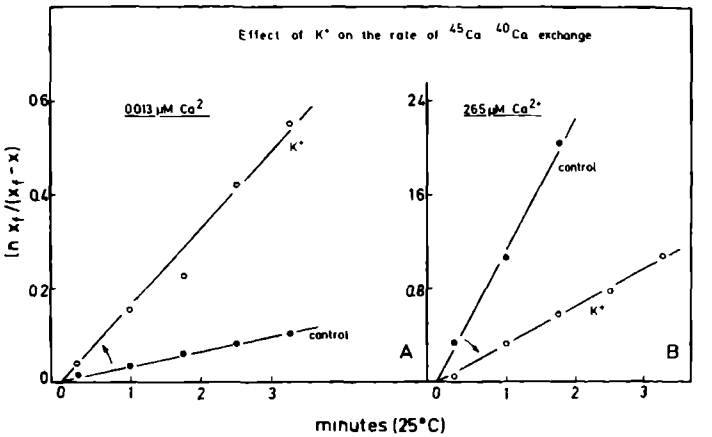

Fig. 7.
Effect of K^+ on the ^{45}Ca-^{40}Ca exchange rate in the standard medium at 25°C. The data are plotted according to equation (1). In addition to the standard medium:
A Ca^{2+} = 0.013 µM; no KCl (-•-); +50 mM KCl (-o-).
B Ca^{2+} = 265 µM, no KCl (-•-); +50 mM KCl (-o-).

On the contrary, the rate of ^{45}Ca-^{40}Ca exchange is increasingly stimulated when the external Ca^{2+} concentration is lowered, and decreased, when the external Ca^{2+} concentration is raised (Figs. 7 and 8). The Lineweaver-Burk presentation, shown in Fig. 8, analyses the relation between the undirectional ^{45}Ca-fluxes (as obtained from plots like shown in Fig. 7) and the external free Ca^{2+} concentration. The control represents a rod suspension in the standard medium (an extended plot from ref. 8). The linear plot, obtained in this case, indicates a simple single-site saturation mechanism. No anomalies are observed,

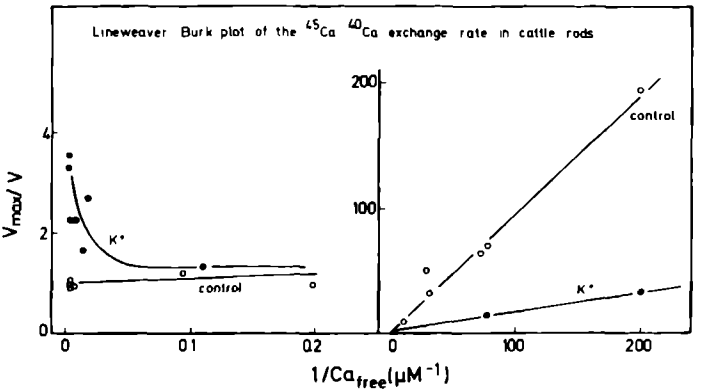

Fig. 8:

Effect of K^+ on the Lineweaver-Burk plot of the unidirectional ^{45}Ca-flux. Data points are obtained from kinetic plots as shown in Fig. 7 and are calculated according to the equations (1) and (2). The data are plotted according to the equation: $v_m/v = 1 + k_{Ca}/Ca^{2+}$, and the same symbols are used as in Table I.

Open symbols: standard medium (data points from the right part of the figure are taken from Ref. 8, Fig. 5)

Closed symbols: in addition to the standard medium 50 mM KCl is added at the start of the incubations and simultaneously with ^{45}Ca.

Temperature 25°C.

when the exchange system is either fully saturated (Fig. 8 left, external Ca^{2+} concentrations up to 300 µM as compared with an affinity for Ca^{2+} of 1 µM), or, when the exchange system is nearly completely unoccupied (Fig. 8 right, free external Ca^{2+} concentrations below 0.01 µM). Simultaneous addition of ^{45}Ca and K^+ ions apparently increases the affinity for Ca^{2+} ions from 1 µM to 0.16 µM concomitant with a reduction of the maximal rate of exchange at saturating Ca^{2+} concentrations. Upon prolonged exposure to high K^+ concentrations the original situation (i.e. similar to that in the absence of K^+ ions) seems restored in both respects (Ref. 8, Fig. 5), suggesting that the above described effects of K^+ ions are transient.

Effects of the combined addition of monovalent and divalent cations

The last part of the previous paragraph described that K^+ ions affect the exchange diffusion system of rods not in a competitive, but in a rather indirect way. Simultaneous addition of K^+ ions and ^{45}Ca in the presence of 250 µM external Sr^{2+} affects the rate of ^{45}Ca-^{40}Ca exchange in a way comparable to that in the presence of an additional 250 µM Ca^{2+} (Fig. 7B). In the presence of an external 250 µM Sr^{2+} the ^{45}Ca-^{40}Ca exchange rate is reduced to 23% by K^+ ions (4 observations, S.E.=2).

In the presence of divalent cations, incapable of stimulating ^{45}Ca-efflux (Fig. 1B), K^+ ions have a different effect, opposed to that in the presence of the transportable ions Ca^{2+} and Sr^{2+}. Fig. 9 shows that K^+ ions increase the

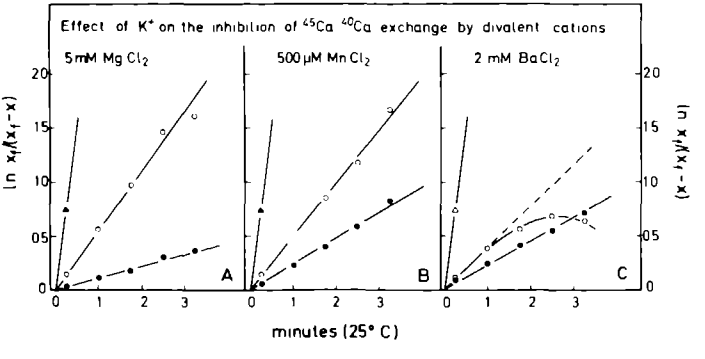

Fig. 9:
Effect of K^+ on the inhibition of the ^{45}Ca-^{40}Ca exchange rate by divalent cations at 25°C. The data are plotted according to equation (1). In addition to the standard medium:
A no additions (-▲-); 5 mM $MgCl_2$ (-●-); 5 mM $MgCl_2$ + 50 mM KCl (-o-)
B no additions (-▲-); 500 µM $MnCl_2$ (-●-); 500 µM $MnCl_2$ + 50 mM KCl (-o-)
C no additions (-Λ-); 2 mM $BaCl_2$ (-●-); 2 mM $BaCl_2$ + 50 mM KCl (-o-)
The external Ca^{2+} concentration amounted to 50 µM.

^{45}Ca-^{40} exchange rate in the presence of non-transportable divalent cations. This is consistent with the previous observation (Fig. 8) that K^+ ions increase the affinity (i.e. decrease the dissociation constant) of Ca^{2+} ions to the common site of the exchange diffusion system.

In a previous section it was shown that the rod cation translocation system discriminates cations into two classes, most probably representing ions transportable, resp. non-transportable by exchange diffusion with Ca^{2+}. Fig. 10

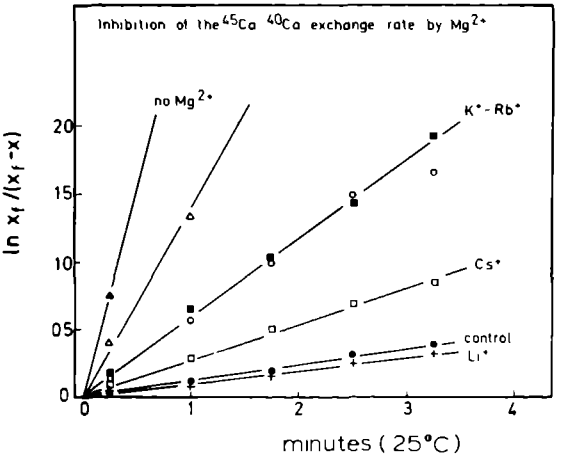

Fig. 10:
Effect of monovalent cations on the inhibition of the ^{45}Ca-^{40}Ca exchange rate by Mg^{2+} at 25°C. The data are plotted according to equation (1). In addition to the standard medium containing 5 mM $MgCl_2$: no additions (-●-); 50 mM LiCl (-+-); 50 mM KCl (-○-); 50 mM RbCl (-■-); 50 mM CsCl (-▽-); $MgCl_2$ omitted (-▲-); 160 mM KCl replaces 480 mM sucrose + 4% Ficoll 400 in the standard medium (-Δ-) and the rods are preincubated for 10 minutes in this KCl-medium before addition of Mg^{2+} and ^{45}Ca. The external Ca^{2+} concentration amounted to 50 µM.

reveals a second discriminatory criterion: K^+, Rb^+ and to a lesser extent Cs^+ ions all appear to change the relative affinities of Mg^{2+} and Ca^{2+} (as well as of the other divalent

cations) for the common site on the exchange diffusion system, whereas Li^+ ions, Tris (not shown) and all divalent cations are most likely ineffective.

A remarkable feature is observed in Fig. 9C. The kinetic analysis of ^{45}Ca-equilibrium in the presence of both K^+ and Ba^{2+} ions gives a linear plot only during the first minute of the incubation. The curious curvature of the plot is explained by the observations, shown in Fig. 11. Whereas Ba^{2+} ions and

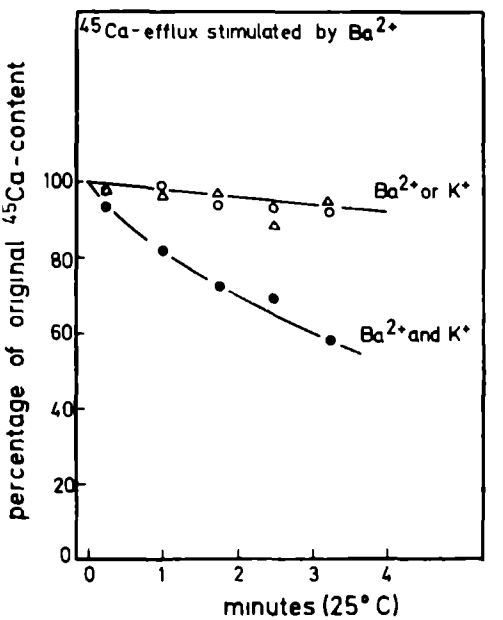

Fig. 11:
Ba^{2+}-stimulated ^{45}Ca-efflux from rods preloaded in the standard medium at 25°C. The efflux experiment is started by the addition of: 10 mM KCl (-Δ-); 5 mM $BaCl_2$ (-o-); 10 mM KCl + 5 mM $BaCl_2$ (-•-) The data are expressed as the percent with respect to the ^{45}Ca-level after the previous equilibration.

K^+ ions separately do not stimulate ^{45}Ca-efflux (see also Fig. 1), addition of both ions together results in a ^{45}Ca-efflux, but at a slower rate that observed for ^{45}Ca-^{40}Ca exchange. Potassium ions appear to bring about a conformational change of the exchange diffusion system, which then allows

for transport of Ba^{2+} ions. The alternative interpretation, i.e. Ba^{2+}-stimulated K-Ca exchange is possible as well, but seems not very likely.

Effects of Na^+ ions: competition with Ca^{2+} ions for a common site

In a previous paragraph it was suggested (Fig. 6A) that Na^+ ions competitively inhibit the ^{45}Ca-^{40}Ca exchange rate. The ^{45}Ca-^{40}Ca exchange rate is inhibited by external La^{3+} ions (8) and external Mg^{2+} ions (Fig. 5). ^{45}Ca-efflux, stimulated by external Na^+ ions (Fig. 1A), most likely represents Na-Ca exchange, by which external Na^+ and Ca^{2+} act on a common external site of the exchange diffusion system. These observations lead to the suggestion that La^{3+} ions and Mg^{2+} ions will reduce the rate of $\overset{+}{Na}$-stimulated ^{45}Ca-efflux from rods, whereas addition of external EGTA is expected to increase the rate of $\overset{+}{Na}$-stimulated ^{45}Ca-efflux from rods. The experiments shown in Fig. 12 confirm these expectations and are consistent with the fact that external Na^+ and Ca^{2+} compete for a common external site of the exchange diffusion system. Because of complications described in the next paragraph a precise kinetic analysis is not feasible. Tentative calculations on data as shown in Fig. 12 result in values for the affinity of Na^+ ions to the exchange diffusion system, which range between 1-3 mM. For these calculations it is assumed that Na-Ca exchange is electroneutral (stoichiometry of 2 Na^+ - 1 Ca^{2+}).

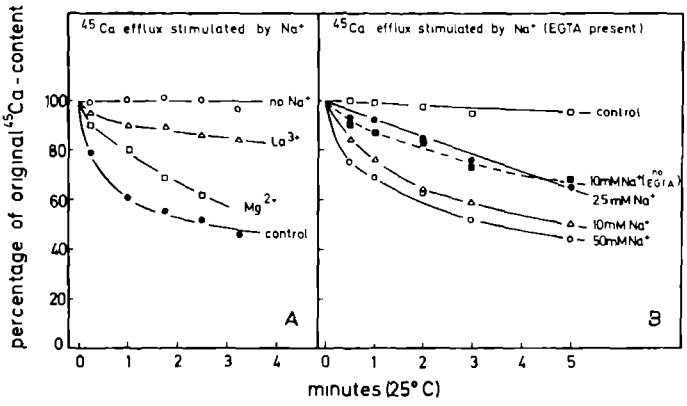

Fig. 12:

Properties of Na^+-stimulated ^{45}Ca-efflux *from preloaded rods* in the standard medium at 25°C. ^{45}Ca-efflux is started by addition of.
A no additions (-o-); 50 mM NaCl (-•-); 50 mM NaCl + 250 µM $LaCl_3$ (-Δ-); 50 mM NaCl + 5 mM $MgCl_2$ (-□-).
B 100 µM EGTA (-▫-); 100 µM EGTA + 2.5 mM NaCl (-•-); 100 µM EGTA + 10 mM NaCl (-Δ-); 10 mM NaCl, no EGTA (-■-); 100 µM EGTA + 50 mM NaCl (-o-).
The data are expressed as the percent of the ^{45}Ca-level after the previous equilibration.

Effects of Na^+ ions: Na^+ ions make endogenous Ca^{2+} inaccessible to rapid Ca-Ca exchange

It was previously found that external ^{45}Ca exchanges completely with endogenous Ca^{2+} in a process, which could be described by a single rate constant (8). A similar behaviour is also observed in this study as indicated by the linear plots in Fig. 7,9 and 10. However, in spite of the presence of EGTA no complete ^{45}Ca-efflux can be obtained with external Na^+ ions (Fig. 1A and Fig. 12B). After a rapid initial efflux phase lasting about 1 minute ^{45}Ca-efflux fades away, although no equilibrium can have established at the common external site of the exchange diffusion system. This behaviour is

investigated in detail in the experiment shown in Fig. 13.

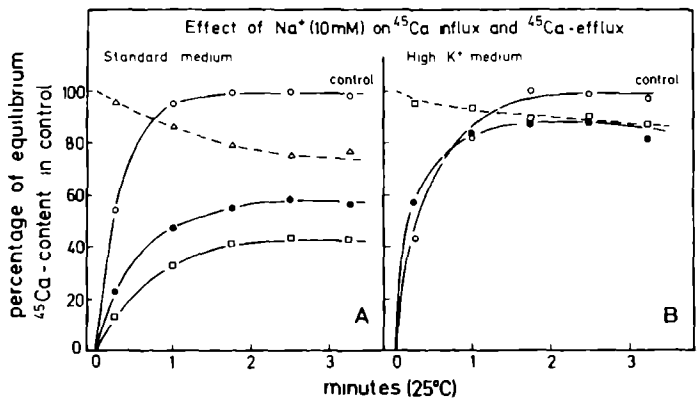

Fig. 13:
Effect of Na^+ on the equilibrium ^{45}Ca-level at 25°C.
A In addition to the standard medium:
no additions (-o-); 10 mM NaCl (-•-); 10 mM NaCl added 3 minutes before addition of ^{45}Ca (-□-); 10 mM NaCl after previous equilibration with ^{45}Ca (-Δ-).
B Same symbols as in A represent the same additions.
160 mM KCl replaces 480 mM sucrose + 4 Ficoll 400 in the standard medium and the rods are 10 minutes preincubated in this medium before the other additions start the incubations.
The data are presented as the percent with respect to the ^{45}Ca-level after equilibration without Na^+.

After prior equilibration with ^{45}Ca, Na^+ ions stimulate ^{45}Ca-efflux from rods to a level, which is not reached, when ^{45}Ca and Na^+ are added simultaneously. A 3 minute preincubation with Na^+ and subsequent addition of ^{45}Ca does not change the result (the reduction of the equilibrium level of ^{45}Ca-uptake after preincubation with Na^+ as compared to that without preincubation is accounted for by the ^{45}Ca-efflux during the preincubation period; Fig. 13A, broken line). These observations mean that in the presence of

external Na^+ ions the endogenous Ca^{2+} pool is not longer homogeneous. Endogenous Ca^{2+} is now divided into two pools as opposed to the single pool observed under the Na^+-free conditions used in Figs. 7,9 and 10 (see also Ref. 8, Figs. 4 and 6). One pool exchanges with external ^{45}Ca at a normal rate, whereas the other pool does not exchange with external ^{45}Ca at a noticeable rate within the time course of the experiment. When the rods are exposed to a medium with a high K^+ concentration, the ^{45}Ca-levels obtained in the influx- and efflux experiment coincide without any indication for an inhomogeneity of the endogenous Ca^{2+} pool in spite of the presence of external Na^+ ions (Fig. 13B). In addition , K^+ ions seem to change the relative affinities of the exchange diffusion system for Ca^{2+} and Na^+ ions resp. This is indicated by the reduced ^{45}Ca-efflux, stimulated by Na^+ (10 mM) in the presence of K^+ ions (Fig. 13). This effect is consistently observed at Na^+ concentrations < 20 mM.

From the foregoing it is obvious that Na^+ ions and K^+ ions have complicated and interrelated effects on ^{45}Ca-fluxes in rods. These effects become particularly prominent and unambiguous at elevated external Ca^{2+} concentrations. Under these conditions the competitive effects of Na^+ ions on ^{45}Ca-uptake and therewith net Ca^{2+} transport are minimized. Fig. 14A shows that an increasing part of the endogenous Ca^{2+} in rods become inaccessible to rapid exchange with external ^{45}Ca when the external Na^+ concentration is gradually raised. The kinetic analysis shown in Fig. 14B

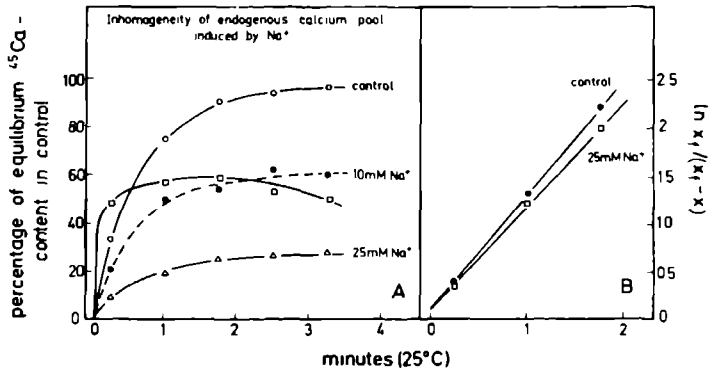

Fig. 14:

Effect of Na^+ on the accessibility of endogenous Ca^{2+} to rapid exchange with external ^{45}Ca at 25°C.
To a rod suspension in the standard medium external Ca^{2+} is added to a final external Ca^{2+} concentration of 250 µM. To this suspension are added:

A no additions (-o-); 10 mM NaCl (-•-); 25 mM NaCl (-Δ-);
25 mM NaCl + 10 mM KCl (-□-).
The data are expressed as the percent with respect to the ^{45}Ca-level after equilibration without added Na^+ or K^+.

B Kinetic analysis of the ^{45}Ca-^{40}Ca exchange rate of exchangeable calcium with and without 25 mM NaCl.
The data are plotted according to equation (1) and present the average of three determinations. In this case X_f represents the equilibrium ^{45}Ca-level, which is not identical with the ^{40}Ca-level, when Na^+ ions are present.
no additions to the standard medium (-•-); 25 mM NaCl added simultaneously with ^{45}Ca at the start of the incubation (-□-).

demonstrates that the endogenous Ca^{2+} pool in rods, which still exchanges with external ^{45}Ca, does so with nearly the same rate irrespective of the presence of Na^+ ions. This indicates that under these conditions external Na^+ ions have only marginal competitive effects on the ^{45}Ca-^{40}Ca exchange rate.

Within the concentration ranges used, no other cation tested in this study is capable to substitute for Na^+ ions

with respect to the ability to induce inhomogeneity of the endogenous Ca^{2+} pool in rods. K^+ ions, however, appear to act as antagonists of Na^+ ions and restore the accessibility of the endogenous Ca^{2+} pool to exchange with external ^{45}Ca (Fig. 14, see also Fig. 13).

In the Figs. 7 and 8 it was shown that K^+ ions decrease the maximal rate of ^{45}Ca-^{40}Ca exchange in the presence of high external Ca^{2+} concentrations (200-300 μM). In Fig. 14A it is observed that the combined addition to the rod suspension of Na^+ and K^+ ions in the presence of a high external Ca^{2+} concentration increase the maximal rate of ^{45}Ca-^{40}Ca exchange considerably.

In the presence of external Na^+ ions, ^{45}Ca-fluxes in rods are affected in three ways by external K^+ ions. Firstly, the accessibility of endogenous Ca^{2+} to rapid exchange with external ^{45}Ca is restored (Fig. 14). Secondly, the maximal rate of Ca-Ca exchange is increased (Fig. 14). Finally, the relative affinities of the exchange diffusion system for Na^+ and Ca^{2+} ions are changed (Fig. 13, efflux curves). Li^+ ions and protonated tris cannot substitute for K^+ ions in these effects.

Effects of Na^+ ions: Na^+ ions make endogeneous Ca^{2+} inaccessible to rapid Na-Ca exchange

External Na^+ ions make part of the endogenous Ca^{2+} in rods inaccessible to rapid exchange with external ^{45}Ca (previous paragraph). The observations shown in Fig. 15 indicate that external Na^+ ions induce a similar

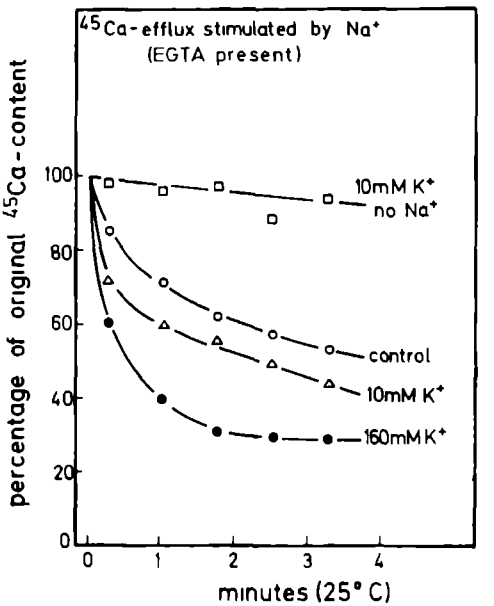

Fig. 15:

Effect of Na^+ on the accessibility of endogenous Ca^{2+} to rapid Na^+-stimulated ^{45}Ca-efflux at 25°C.
Rods are equilibrated at 25°C in the standard medium with ^{45}Ca, or in one case in a medium, in which 160 mM KCl replaces 480 mM sucrose + 1% Ficoll 400 (-●-). The incubations are started by addition of 250 µM EGTA and:
10 mM KCl (-□-); 50 mM NaCl (-o-); 10 mM KCl + 50 mM NaCl (-Δ-), and to the rods in the KCl-medium: 50 mM NaCl (-●-).
The data are expressed as percent with respect to the ^{45}Ca-level after the previous equilibration.

inaccessibility of the endogenous Ca^{2+} pool (previously marked by ^{45}Ca) to exchange with the same Na^+ ions. At high external Na^+ concentrations the amount of endogenous Ca^{2+} accessible to rapid Na^+-stimulated Ca^{2+}-efflux is increased upon addition of external K^+ ions in a similar way as observed for Ca-Ca exchange in Fig. 14. At lower Na^+ concentrations (< 20 mM) the change of the relative

affinities, induced by K^+ ions and resulting in a preference for Ca^{2+} with respect to Na^+, predominates and causes a reduced rate of Na^+-stimulated Ca^{2+}-efflux upon addition of K^+ ions (Fig. 13, efflux curves).

Osmotic experiments

An essential prerequisite for the interpretation of the effects of the various tested electrolytes on ^{45}Ca-fluxes in rods is to establish which of the electrolytes may permeate through the rod membrane in another way than indicated by rapid exchange with endogenous Ca^{2+}. In other words, which externally applied electrolytes may accumulate to a considerable extent in the rod cytosol and therefore may act also on the disk membranes.

The turbidity of a rod suspension is used as an assay for osmotic shrinkage and swelling. With the optical geometry of the two spectrophotometers used, addition of 100 mosmolar (impermeable) electrolyte to a rod suspension in the threefold diluted standard medium results in a 10-20% increase of the apparent absorbance at 700 nm (0.05-0.1 optical density) indicating a shrinkage of the rods. The first row of Table II shows that upon addition of permeable electrolytes like ammonium acetate or alkali cation acetates in the presence of Gramicidin D the absorption changes are rapidly recovered. This result supports the validity of the method used.

TABLE II

The recovery of intact cattle rods from a hypertonic shock, monitored by light-scattering changes

applied electrolyte (100 mosmolar)	fast recovery (< 1 minute)	comments
NH_4Ac	100%	
LiAc, Gramicidin D present (3 μM)	100%	
NaAc, Gramicidin D present (3 μM)	100%	
KAc, Gramicidin D present (3 μM)	100%	
LiCl	< 10%	slow recovery: < 1% min
NaCl	< 10%	slow recovery: 4.5 ± 0.5 (8)
KCl	< 10%	slow recovery: < 1% min
NH_4Cl, FCCP present (5 μM)	20 ± 3 (3)	slow recovery: < 1% min
NaCl, Gramicidin D present (3 μM)	23 ± 4 (3)	slow recovery: < 1% min
NaAc,KAc,LiAc	29 ± 3 (7)	further slow recovery
LiAc, FCCP present (5 μM)	55 ± 3 (3)	
NaAc, FCCP present (5 μM)	79 ± 9 (4)	
KAc, FCCP present (5 μM)	75 ± 9 (4)	
$CaAc_2$,FCCP present (5 μM)	< 10%	
$MgAc_2$,FCCP present (5 μM)	< 10%	
$CaAc_2$,A23187 present (5 μM)	61 ± 7 (5)	slow increase of absorbance

TABLE II (continued)

A rod suspension in the standard medium is diluted immediately before use such as to yield a threefold reduction of the osmotic strength (final medium : 200 mM sucrose, 5/3% w/v Ficoll 400, 10-20 mM tris HCl at pH=7.4). Light-scattering is followed at room temperature the 5 minutes preceding the hypertonic shock (increase by 100-mosmolar) and the 5 minutes following the hypertonic shock. The data are presented as the percent of the initial increase of apparent absorption, when a relatively impermeable electrolyte is used. The rate of slow recovery is expressed as percent per minute and is presented as the mean ± standard error and the number of observations between parentheses.

The second row of Table II shows that alkali cation chlorides do not permeate (the origin of the slow recovery with NaCl is unclear). This result implies that the plasma membrane of the cattle rod preparation does not contain conductance pathways for both alkali cations and chloride ions. When the conductance barrier for the cations is removed by the addition of appropriate ionophores(Gramicidin D, NH_4Cl in combination with FCCP) still no large recoveries are observed (third row in Table II). This suggests that the plasma membrane of rods is impermeable to chloride. The conductance barrier for anions can be removed by the use of acetates in combination with FCCP. Acetic acid permeates and the protons go back to the external medium via FCCP if the concerned cation permeates, resulting in a net transport of the cation acetate. The fourth row of Table II shows that a substantial to complete recovery is observed, when alkali cation acetates are used in combination with FCCP, but not for earth alkali cations. In the latter case a recovery for calcium acetate is observed when in combination with the use of acetates the exchange carrier A23187 is included. In this case acetic acid permeates and the protons return to the external solution in A23187 mediated exchange for external divalent cations, resulting in net transport of calcium acetate across the plasma membrane.

Reproducibility and observations on stable leaky rods

During the course of the work, presented in this study, 43 intact rod preparations were used and 5 preparations of stable leaky rods. All effects shown in the figures of this

report proved to be qualitatively almost always reproducible. Quantitative agreement is within a factor of two to three. Remarkable observations are obtained with the stable leaky rods. Qualitatively these leaky rods behave exactly similar to the intact rods when ^{45}Ca-metabolism is concerned. Experiments similar to those shown in Figs. 9A, 11 and 14 yield identical pictures from simultaneously prepared leaky and intact rods.

In 2 of the 43 intact rod preparations anomalous behaviour was observed. Endogenous Ca^{2+} did not or very slowly exchange with external ^{45}Ca ($t > 10$ minutes as compared with the normal 12 seconds). Analogous to Fig. 14, external K^+ ions restore exchange, i.e. relieve the inaccessibility of the endogenous Ca^{2+} pool in these preparations. This effect is again specific for K^+ ions. Neither Na^+ ions, Ca^{2+} nor Mg^{2+} ions can substitute for K^+ ions.

DISCUSSION

Experiments on the electrical behaviour of the vertebrate retina have been until now the major source of information about the ion fluxes underlying excitation of vertebrate rod photoreceptor cells. Measurements by intracellular (4) and extracellular recording techniques (2,12) have indicated the presence of a cation translocation system in the rod outer segment plasma membrane, which is capable to discriminate Na^+ ions from all other cation tested and which can carry a net current of Na^+ ions. Ca^{2+} ions, but not Mg^{2+} ions, can inhibit this Na^+ current (1,4), but cannot replace Na^+ ions as charge carriers. In addition, the rod outer segment plasma membrane presumably contains a conductance pathway for K^+ ions (1,5) and may contain voltage-dependent conductance pathways (13,14). As compared to the permeability for Na^+ ions the permeability for Cl^- ions appears to be low (4,15). To extend this information to the molecular basis of these electrical phenomena direct measurements of ion fluxes with well-defined rod outer segment preparations may be useful.

Following the strategy outlined in two previous communications (7,8), this study intends to give a general survey of the ion selectivity of the translocation system(s) present in the plasma membrane of isolated cattle rods rather than to provide a fully detailed picture. Furthermore, the necessity to impose rather unphysiological conditions on isolated rod outer segments during purification and storage,

urges to caution. On the other hand, qualitative reproducibility and quantitative homogeneity of the rod preparations used strengthen confidence in the results obtained and do not indicate a significant contribution of contaminating material.

The two types of experiments described in this study appear to provide complementary information about the ion fluxes through the rod plasma membrane of the intact cattle rods used. The osmotic experiments reveal electrogenic uniport or electroneutral symport, whereas the ^{45}Ca-experiments describe electroneutral antiport.

Osmotic experiments

The control experiments with the intact rods and the permeable electrolytes (first row in Table II) support the validity of the osmotic technique used. This implies that the rod cytosol is the osmotically active compartment observed. The plasma membrane of intact rods is found to be relatively permeable to net transport of Li^+, Na^+ and K^+ ions, and relatively impermeable to net transport of Cl^-, Mg^{2+} and Ca^{2+} ions. Only in the presence of an external divalent cation carrier (A23187) net Ca^{2+} transport does occur in agreement with the ^{45}Ca-experiments described before (8). As a consequence of the relative impermeability of the rod plasma membrane to Cl^- ions as compared to the permeability to Li^+, Na^+ and K^+ ions, chloride salts of the latter cations do not permeate through the plasma membrane to an appreciable extent, but may give rise to

diffusion potentials across the plasma membrane. At an external Na^+ concentration of 50 mM net transport of 8×10^5 positive charges through the plasma membrane of a cattle rod would generate a diffusion potential of 160 mV (inside positive) concomitant with an increase of the rod cytosol Na^+ concentration of 0.08 mM. Dimensions of 1×20 µM and a volume of 16 fl are used for a cattle rod outer segment, and a membrane capacitance of 1 $\mu F/cm^2$ is assumed. A complete recovery from a hypertonic shock with 50 mM NaCl would require an influx of 10^9 particles (Na^+ or Cl^- ions) into the rod cytosol. The impermeability of the rod plasma membrane to chloride salts due to the absence of a Cl^- conductance conductance pathway is in agreement with electrophysiological observations (4,15) and with observations on isolated frog rod outer segments by some authors (16,17). Others however do report that isolated frog rod outer segments are permeable to Cl^-, Na^+ and Li^+ ions, but not to K^+ ions (18,19,20). The reason for this discrepancy is unclear.

^{45}Ca-fluxes: interpretation of transport mode

An essential conclusion to be drawn from the osmotic experiments with cattle rods is that all the electrolytes (chloride salts) used in the ^{45}Ca-experiments do not permeate to any extent through the plasma membrane during the incubation times used and are only exposed to the external side of the plasma membrane. Therefore, they may interfere with ^{45}Ca-fluxes in intact rods only by acting on the exchange diffusion system in the plasma membrane either directly

(competition for an external site) or indirectly by establishing a diffusion potential across the plasma membrane. No substantial amount of the externally applied chloride salts can accumulate inside rods otherwise than indicated by stimulation of ^{45}Ca-efflux by exchange diffusion. Therefore, stimulation of rapid ^{45}Ca-efflux inevitably reflects electroneutral exchange if no other charge carriers are available (e.g. cytosol K^+ ions) and in the absence of ion pumps (8). In summary, it is concluded that the experiments shown in Fig. 1, 11, 12 and 15 represent, at least for the greater part, electroneutral Ca-Ca, Ca-Sr, Ca-Ba and Ca-2Na exchange. This conclusion has been explicitely confirmed previously (8) for the case of Ca-Ca exchange.

Properties of the exchange diffusion system in the rod plasma membrane

An analysis of the ^{45}Ca-fluxes in intact isolated cattle rods reveals that the exchange diffusion system present in the rod plasma membrane contains at least four distinct functions:

1- A low selectivity external binding site, which determines the affinity towards transport, but not the actual transport rate. The selectivity order is:
$La^{3+} > Mn^{2+} > Ca^{2+} > Sr^{2+} > Ba^{2+} > Mg^{2+}$ and
$Na^+ > Li^+ > tris^+$; $K^+ \sim Rb^+ \approx Cs^+ \approx 0$
This common site determines competitive inhibition of the ^{45}Ca-^{40}Ca exchange rate as shown in the Figs. 3, 5 6, 9, 10 and 12. Further evidence that the low-selecti-

vity site is an independent entity of the exchange diffusion system comes from the La^{3+}-effects described before (Ref. 8 Table IV). Apparently, this site can be disconnected from the actual transport function, in view of the loss of inhibitory effects by La^{3+} without a parallel loss of exchange transport.

2- The conformational state of the exchange diffusion system, which results from the binding of cations to the low-selectivity site subsequently discriminates with high selectivity, which cation may actually be transported. There is clearly no correlation between the affinity for the common binding site (Table I) and actual transport (Figs. 1 and 11). Ca^{2+}, Sr^{2+} and Na^{+} ions are transported at approximately the same maximal rate. Ba^{2+} and (possibly) Li^{+} ions may be transported under certain conditions (Figs. 1, 2, 3 and 11), but at a lower rate, whereas for the other tested cations the actual transport rate appears to be practically zero.

3- The observations shown in Figs. 13, 14 and 15 indicate a third function with an unique selectivity for Na^{+} ions. This function operates as an on-off switch for the exchange diffusion transport mode. Any model based on the reversible binding of Na^{+} ions to a certain site which then operates as a switch would result in a stochastic closure and opening of all transport entities during the incubation time and consequently in a reduced, but uniform transport rate. This is evidently not the case.

4- A fourth function, like the third not of competitive nature, is revealed by the observations shown in Fig. 10 and has a complementary selectivity with respect to the low-selectivity site. Fig. 10 and its discussion demonstrate that only K^+, Rb^+ and Cs^+ ions, which appeared to have no affinity towards the common low-selectivity site, may modulate the exchange diffusion system. This has been extensively investigated only for K^+ ions, but it is assumed that Rb^+ and Cs^+ ions behave similar (as shown in Fig. 10). All effects of external K^+ ions are presumably due to this function, which is rather versatile. Firstly, the Lineweaver-Burk plot (Fig. 8) suggests that K^+ ions alter the absolute affinity of the exchange diffusion system for Ca^{2+} ions. Therefore the results shown in Figs. 9 and 13 (efflux curves) are consistent with the fact that the relative affinities for all cations are changed. Secondly, external K^+ ions may control the maximal transport rate of Ca-Ca exchange (Figs. 7, 8 and 14) and the range of ions, which are transported (Fig. 11). Finally, external K^+ ions act as antagonists of Na^+ ions with respect to the operation of the third function.

The fourth function may indicate the existence of a K^+-sensitive site(s) on the exchange diffusion system. Alternatively, K^+ ions may be expected to establish a diffusion potential. This would mean that the first three functions described here are all dependent on the membrane potential.

In none of the experiments, described in this study, external energy sources are added. Thus, a direct and causal relation between hydrolysis of ATP and all the ^{45}Ca-fluxes and transport functions described in this study can be excluded by an identical line of reasoning as used before (8). Intact cattle rods, however, appear to contain sufficient high-energy phosphates (7) and a more indirect involvement seems possible, but proves difficult to substantiate (Schnetkamp, P.P.M., unpublished material).

Comparison of the rod exchange diffusion system with other exchange systems

Cation exchange systems, which are selective for i.a. Na^+ and Ca^{2+} ions are described for a number of tissues (for a review, see Ref. 21). Similar properties as described here for the first two functions of the rod exchange diffusion system are found for the Na-Ca exchange system, present in giant squid nerve axons (22,23). Also the Ca-Ca exchange, described for rat heart mitochondria (24,25), has similar properties as the rod exchange diffusion system. However, in the latter case a different ion selectivity has to be noted. Rat heart mitochondria do not discriminate between Li^+ and Na^+ ions, and have a tenfold lower affinity towards Ca^{2+} ions (25). Furthermore, the mitochondrial Ca^{2+} pool is sustained by energy-requiring processes (25), whereas the rod Ca^{2+} pool is stored by binding (8).

Comparison of the exchange diffusion system with electrophysiological data

Although care should be taken to extrapolate data obtained from isolated cell organelles to the *in vivo* situation, the exchange diffusion system, present in the plasma membrane of isolated intact rods and described in this and the previous study (8), has a number of properties in common with the system responsible for the dark current of Na^+ ions in the vertebrate retina.

1- The exchange diffusion system resides in the plasma membrane of isolated cattle rods and responds to a disturbance of the external ionic conditions (in particular the Ca^{2+}/Na^{2+} ratio) by changing the intracellular concentrations of the respective ions with half-times in the order of 10-60 s (Figs. 1,12-15). Similarly, the intact rod photoreceptor cell in the retina responds rapidly to changes in the external medium and inhibition of the ionic battery (1), which are interpreted by concomitant changes of the intracellular concentrations of the respective ions (notably Na^+ and Ca^{2+} ions, Refs. 1 and 4).

2- Rod photoreceptor cells in the retina respond electrically quite different to external Li^+, Na^+ or K^+ ions. At a normal external Ca^{2+} concentration (1,36 mM) Li^+ ions cannot replace Na^+ ions as charge carriers in the dark current. The hyperpolarization of the rod photoreceptor cell upon substitution of Li^+ ions for Na^+

ions indicates that the permeability of the rod plasma membrane for Li^+ ions is much lower than that for Na^+ and K^+ ions (2,4,12). Variation of the external K^+ concentration modulates both the dark membrane potential and the photoreceptor potential (4,12). The exchange diffusion system of isolated rods also discriminates sharply between external Li^+, Na^+ and K^+ ions. Na^+ ions are efficiently transported, Li^+ ions are mainly inert and K^+ ions may establish a diffusion potential, but in any case exert strong effects not of competitive nature (Figs. 1,8,10 and 14).

3- In the retina, variation of the external Na^+ and Ca^{2+} concentration modulates the dark current, the membrane potential and the photoreceptor potential (1,2,4,6) in a way, which can be understood in terms of competition between Na^+ and Ca^{2+} ions for a common site. Upon reduction of the external Ca^{2+} concentration a dark current can be measured with external Na^+ concentrations as low as 1 mM (2). Likewise, Ca^{2+} and Na^+ ions appear to compete for a common site on the exchange diffusion system of isolated cattle rods (Figs. 6 and 12). At low external Ca^{2+} concentrations an affinity of the exchange diffusion system of 1-3 mM to Na^+ ions enables Na^+ transport at low Na^+ concentrations (Fig. 12).

4- The affinity of Ca^{2+} ions towards the system, responsible for the dark current of Na^+ ions in the retina has been calculated to be 1 µM (3). The affinity of the exchange diffusion system of isolated cattle rods is found to be adjustable between 0.16-1 µM (Fig. 8).

5- The plasma membrane of isolated cattle rods is found to be able to carry a net current of Na^+ ions, but not of Ca^{2+} ions (Table II). The dark current of Na^+ ions in the retina is found to be inhibited by Ca^{2+} ions, but Ca^{2+} ions cannot replace Na^+ ions as charge carriers (1,2,4,6).

6- The dark current of Na^+ ions in rods in the rat retina is measured to amount 20-70 pA/rod or $1-3 \times 10^{14}$ charges/cm^2/sec. (2). The maximal Ca-Ca exchange rate observed for isolated cattle rods in this study (conditions as in Fig. 14 in the presence of both Na^+ and K^+ ions) amounts to 0.1×10^{14} (Ca-Ca)/cm^2/sec. or 0.4×10^{14} charges/cm^2/sec.

7- In the absence of ion pumps in the outer segment (8,26) Na-Ca exchange across the plasma membrane, driven by a Na^+ gradient, would be sufficient for Ca^{2+}-homeostasis in the rod cytosol (cf Ref. 27). A driving potential across the plasma membrane of 60 mV is required for Na^+ ions to maintain a cytosol Ca^{2+} concentration of 1 µM at an external Ca^{2+} concentration of 1.36 mM, when the exchange system operates with a stoichiometry of 3 Na: 1 Ca.

One essential piece of information is obviously missing in this series. It is not shown that net Na^+ transport through the plasma membrane of isolated intact cattle rods is performed by the same system, which is responsable for exchange diffusion transport. Furthermore, in contrast with ^{45}Ca-experiments the osmotic responses of rods do not

discriminate qualitatively between Li^+, Na^+ and K^+ ions, when acetates are used in combination with FCCP (i.e. under a sort of voltage clamp). A further discussion on these points has to await data on the diffusion potentials, established by chloride salts of the monovalent cations in the isolated intact rods, and on the kinetics of the recovery from hypertonic shocks by alkali cation acetates in the presence of FCCP. The minor effects of chloride salts of Li^+ and tris on ^{45}Ca-fluxes as opposed to the effects of KCl, seem to confirm the suggestion, that the plasma membrane in isolated cattle rods is impermeable to Cl^-.

Evidence for direct communication between disks and plasma membrane

At this stage it is interesting to recall the surprising observations described previously (8). Rods with an intact plasma membrane equilibrate external ^{45}Ca with the endogenous Ca^{2+}-pool as fast as do rods with a leaky plasma membrane, although in the former case all ^{45}Ca first has to pass the plasma membrane (the plasma membrane makes up only a few percent of the total disk membrane surface area). The observations, shown in Figs. 1 and 11-15, demonstrate that endogenous Ca^{2+}, which normally behaves as a homogeneous pool and which is for the greater part localized within disks (8), can be mobilized (Figs. 1,11,12,15) or affected (Figs. 13,14) equally rapidly as ^{45}Ca-equilibration and without a noticeable delay. However, these effects are

caused by ions, which were shown in a previous paragraph to act only on the plasma membrane. The implications of these observations are best illustrated by the experiment shown in Fig. 14. In this experiment it is observed, that a gradual increase of the external concentration of Na^+ ions excludes an increasing amount of intradiskal Ca^{2+} from rapid exchange with external ^{45}Ca. This means that either a number of disks become inaccessible to rapid exchange with external ^{45}Ca, or that individual rods as a whole become inaccessible to rapid exchange with external ^{45}Ca. The latter possibility seems difficult to reconcile with the homogeneity displayed by the rod preparation with respect to other properties of ^{45}Ca-metabolism (i.e. the single rate constants observed in Figs. 7,9 and 10; see also Ref. 8). The former alternative implies that internal Ca^{2+} ions are at least for 70% localized (compare in Fig. 14 ^{45}Ca-uptake in the control with that in the presence of 25 mM Na^+) in discrete intracellular compartments. The latter are most probably identical with the disks. Thus, external Na^+ ions act on a transport system, which has access to the extracellular space and in the presence of external Na^+ discriminates between Ca^{2+} ions localized in different disks. This can be understood if a separate system for every disk performs the observed exchange diffusion transport directly between the extracellular space and the intradiskal space. This would imply that single transport units form a material connection between the plasma membrane

and those disks, which are accessible to rapid exchange diffusion. This conclusion explains the findings that stabilized rods with a leaky plasma membrane behave similarly as intact rods as far as ^{45}Ca-metabolism is concerned. The "Na^+- and Ca^{2+}-selective exchange carrier" localized in the disk membranes in a previous study (10) can also be attributed to the presence of a leaky plasma membrane (see discussion in Ref. 10).

Alternative explanations of the above mentioned observations (notably those in Figs. 11 and 14) require the assumption that endogenous Ca^{2+} is predominantly localized in the rod cytosol and bound to the cytoplasmic side of the disk membranes. Furthermore the observations in Fig. 14 imply that endogenous Ca^{2+} can become so tightly bound that it is inexchangeable, even on a minute time-scale. These stipulations are incompatible with the existing experimental evidence: Ca^{2+} in intact, leaky and lysed rods is inaccessible to chelation by external EGTA, but can be chelated rapidly and completely by EGTA upon addition of the ionophore A23187 (8,10). This demonstrates that in the rod preparations used in this and the previous studies (8,10) no binding sites exist, which can compete with EGTA for Ca^{2+} ions and which allow residence times of bound Ca^{2+} ions larger than the subsecond range.

Not inconsistent with a material connection between the disk membranes and the plasma membrane are electron microscopic studies on the osmotic behaviour of rod outer

segments in the intact retina (28,29). In an electron microscopic study on isolated cattle rods a picture is shown of osmotically shocked rods, which give the impression that the disks are kept together by the plasma membrane (Ref. 30, Fig. 1). This observation is consistent with the observed resistance of the intact rods to osmotic lysis.

In the case of an ion transport system, which connects the disk and the plasma membrane, an electrical coupling resistance between disks and plasma membrane, which according to Penn and Hagins (31) is still permitted by the electrical properties of the plasma membrane, should exceed 2×10^{11} ohm. Assuming that the driving potential for the dark current is 50 mV, that the intensity of the dark current amounts to 50 pA and that a rod contains 500 disks (2,31) a value of 5×10^{11} ohm for each connection is calculated. Therefore, a single connection between each disk and the plasma membrane in the form of a cation transport system with the capacity of the dark current is not incompatible with the electrical properties of the rod plasma membrane.

An alternative to the concept of a diffusable transmitter in the rod cytosol

The arguments put forward in the previous paragraph appear to suggest that individual disks can be connected to the rod plasma membrane by a cation transport system, which is selective to Ca^{2+} and Na^{2+} ions. The concomitant

state of the transport system performs exchange diffusion transport and can be reversibly turned off and on by external Na^+ and K^+ ions respectively (Fig. 14). In the state, which does not perform exchange diffusion transport the intradiskal Ca^{2+}-pool appears isolated from the external Ca^{2+}-pool and possibly also from the cytosolic Ca^{2+}-pool (the latter is suggested by the strongly reduced rate of ^{45}Ca-uptake after lysis of intact rods, cf Ref. 8). If this were true the suggestion is obvious that the "off"-state of exchange diffusion transport represents the "on"-state of (electrogenic) transport between the cytosol and the external medium. An intriguing property of the mechanism regulating the ratio of the respective states is the non-stochastic nature mentioned in a previous paragraph. The data of Fig. 14 indicate the presence of a titratable transition between the two states in dependence of the external Na^+ concentration. In the presence of a moderate concentration of Na^+ ions both states are populated, <u>but individual transport entities do not statistically fluctuate between the two states</u> (the latter would result in a slower, but complete ^{45}Ca-equilibration). This can be understood if individual transport entities would act interdependently. This means, that the information about the (change of) state of an individual transport unit is intercommunicated by a certain number of transport units.

Continuing this line of reasoning the notion arises that the process of visual transduction does not need to be mediated by a diffusable transmitter in the rod cytosol.

Bleaching of a rhodopsin molecule could be communicated within the disk membrane to the transport unit residing in that individual disk. The interdependence of individual transport units subsequently enables a transfer of this information to a certain number of other transport units. This would result in a local spread of a quantal event in the length axis of a rod outer segment. Not inconsistent with such a scheme are observations by Hagins et al. (32) and by Jagger (33,34) that local illumination of a rod outer segment results in a response, which is confined to a limited spread from the illuminated zone. This means, that an intracellular transmitter does not diffuse freely in the length axis of a rod outer segment within the time course of a photoresponse. Therefore, the spread of a transmitter by diffusion in the length axis of a rod is expected to be a major constituent in the time course of a photoresponse. On the basis of kinetic (uniform responses to quantal events) and thermodynamic (heat of activation of photoresponses) arguments Baylor et al. (35,36) have reached the conclusion that diffusion of an intracellular transmitter is <u>not</u> the principal rate-limiting step in the generation of a photoresponse in both rod and cone photoreceptors. Combined, these observations seem difficult to reconcile with the concept of a diffusable transmitter in the cytosol communicating between the bleaching of a rhodopsin molecule in the disk membrane and the subsequent conductance change of the rod plasma membrane. This argument is enforced by the fact, that a steady current of Na^+ ions, which is

assumed to flow through the outer segment, does not allow diffusion barriers in the length axis of an outer segment, which would effectively divide the cytosol in subcompartments.

Concluding remarks

Intact isolated cattle rods contain a cation selective transport system, which resides in the plasma membrane and has a number of properties in common with the system responsible for the dark current of Na^+ ions in the vertebrate retina. It combines a high ion selectivity (e.g. sharp discrimination between Li^+, Na^+ and K^+ ions) with a low heat of activation ($Q_{10} = 1.08$, Ref. 8) and a flux, which is sufficient to turnover the complete Na^+- and Ca^{2+}-content of a rod outer segment within one minute. The system contains different functions with distinctive ion selectivity ranges, which appear much more narrow as those of the common Na^+ and K^+ channels (for reviews on the latter see Refs. 37-39). The transport system exists in two states, whose populations are controlled by a mechanism, which infers interdependence of individual transport entities. One state performs exchange diffusion transport directly between the intradiskal space and the external medium and appears to be remarkably leakproof (net transport of Ca^{2+} three orders of magnitude slower than exchange transport, see Ref. 8).

Acknowledgements

My thanks are due to Dr. F.J.M. Daemen, Dr. W.J. de Grip and Dr. J.J.H.H.M. de Pont for critical discussions during the course of the work and during the preparation of the manuscript. The expert technical assistance and many critical discussion of Mr. A.A. Klompmakers and Mr. P.A.A. Jansen are greatfully acknowledged. This investigation is supported in part by the Netherlands Organization for the Advancement of Basic Research (Z.W.O.), through the Foundation for Chemical Research in The Netherlands (S.O.N.).

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CALCIUM IONS AND VISUAL EXCITATION

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The investigations described in this thesis have been carried out in the Department of Biochemistry, (Director: Prof. Dr. S.L. Bonting), University of Nijmegen, The Netherlands and partly (chapter III of the appendix) in the Max-Volmer-Institute of the Technical University Berlin, Germany. Additional financial support was received from the Netherlands Organization for the Advancement of Basic Research (Z.W.O.), through the Foundation for Chemical Research in the Netherlands (S.O.N.).

CALCIUM IONS AND VISUAL EXCITATION

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE
WISKUNDE EN NATUURWETENSCHAPPEN
AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN
OP GEZAG VAN DE RECTOR MAGNIFICUS
PROF. DR. P.G.A.B. WIJDEVELD
VOLGENS BESLUIT VAN HET COLLEGE VAN DECANEN
IN HET OPENBAAR TE VERDEDIGEN
OP VRIJDAG 29 FEBRUARI 1980
DES NAMIDDAGS TE 2 UUR PRECIES

DOOR

PAULUS PETRUS MARIUS SCHNETKAMP
GEBOREN TE 'S HERTOGENBOSCH

1980
Druk: Stichting Studentenpers Nijmegen

Eenieder die aan het tot stand komen van dit proefschrift heeft meegewerkt wil ik graag van harte bedanken.

Mijn dank gaat in het bijzonder uit naar André Klompmakers en Piet Jansen, die mij met grote bekwaamheid en met prijzenswaardige volharding bij het experimentele werk hebben geholpen. Zonder de vele diskussies, die ik met hen heb mogen voeren, zou dit proefschrift niet in zijn huidige vorm tot stand gekomen zijn.

Chapter III of the appendix is the result of a fruitful cooperation with Dr. U.B. Kaupp and Prof. Dr. W. Junge of the Technical University of Berlin. I greatfully acknowledge their contribution to my understanding of biophysical approaches for investigating biological systems. I hope that, after your invitation to join your research group, our further cooperation will continue to be succesful

Aan mijn ouders

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1. INTRODUCTION

1.1. Topology of rod and cone cells

The vertebrate retina is a photosensitive film, which is capable of covering 8-9 decades of light intensity by only two types of photoreceptors, rod and cone cells. Rods cover the lower part of the intensity scale (twilight vision) and contain only one type of photopigment (rhodopsin). This enables only discrimination of light intensity (black-white vision). Cones cover the upper part of the intensity scale (daylight vision). Three types of cones are known, which contain photopigments, that absorb at different wavelengths and thereby enable not only discrimination of light intensity, but also of the spectral properties of the absorbed light (colour vision). Both rods and cones exhibit a large degree of spatial differentiation (Fig. 1).

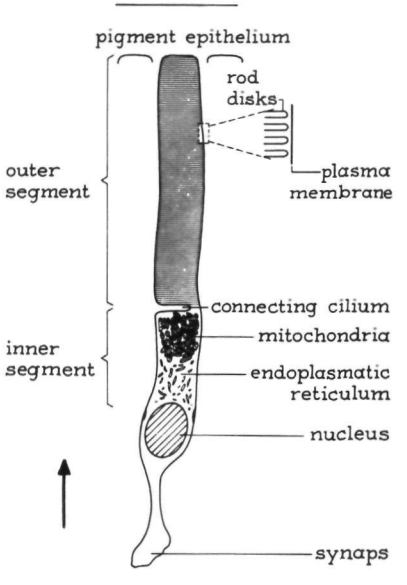

Fig. 1. Schematic diagram of a rod cell. The direction of the incident light is given by the arrow. Modified after Young (1971).

The cell body contains the nucleus and the synaptic terminals. The inner segment is rich in mitochondria and endoplasmic reticulum, and is the site of energy production and protein synthesis. The outer segment is a highly specialized organelle, which contains the photosensitive pigments. It is the site of the transductory and (at least a part of) the adaptive machinery, which mediates between the absorption of a photon and the electrical response of the cell. In most vertebrate retinae, including bovine retinae used in this study, the rod cells greatly outnumber the cone cells. Therefore, mass isolation of rod outer segments is possible from these retinae and nearly all experimental data on isolated outer segments are obtained with rod outer segments. Because of the close parallel between rods and cones with respect to their electrical properties and because cones appear to behave somewhat simpler, the discussion presented here will also extensively draw upon the body of data available on cones.

The major topological difference between rod and cone cells lies in the structure of their outer segments (Fig. 2). In cones, the photopigment molecules are embedded in numerous invaginations of the plasma membrane forming

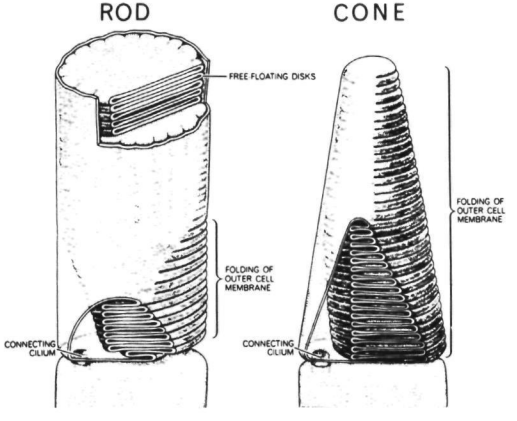

Fig. 2. Schematic presentation of the rod and cone outer segments. The number of disks has been reduced strongly for the sake of clarity. From Young (1970).

a continuous membrane surface directly exposed to the extracellular medium. In rods, the photopigment molecules are embedded in numerous flat saccular structures (Fig. 2) with rather similar appearance as the invaginations of cones (Fig. 3), but enclosed by and not continuous with the plasma membrane. For both rods and cones these flat membrane structures are referred to as disks, and the number of disks amounts to 400-2000 depending on the species.

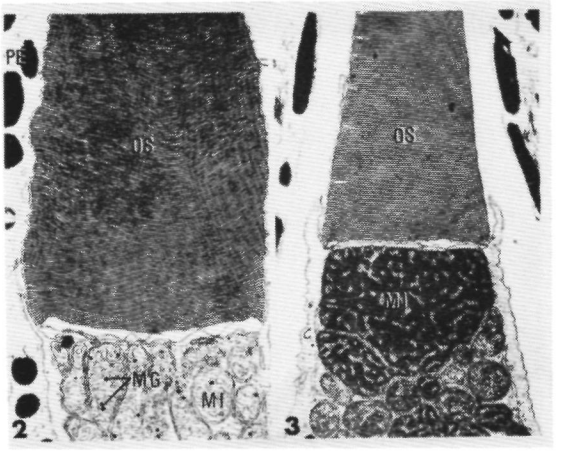

Fig. 3. Electromicrographs of longitudinal sections of part of the rod cell (left) and of part of the cone cell (right) of the guppy (Poecilia reticulata). OS = outer segment, MI = mitochondrion, MG = mitochondrial granule, PE = pigment epithelium. Magnifications : 8000x (left) and 7000x (right). Taken from Yacob et al. (1977).

It has been argued (notably Penn and Hagins, 1972) that in rods the stacked pile of disks is free-floating in the cytosol without direct contacts with the plasma membrane, buth in this investigation (Schnetkamp, 1980) a specific type of contact is described. It is important to note that the space between the disks of cones is in direct contact with the extracellular space, but that the corresponding space in rod disks is separated from the extracellular space by two membranes (disk- and plasma membrane) and a thin zone of the rod cytosol.

For further details on the structural organization of the retina (Cervetto and Fuortes, 1978), the composition of the rod outer segments (Daemen,1973) and the structure of the rod photopigment rhodopsin (Hubbell and Bownds, 1979) the reader is referred to the indicated reviews.

1.2. Aim of this investigation

The aim of this investigation was to find biochemical correlates to the electrical behaviour of rod cells with a special emphasis on the role of Ca^{2+} ions in modulating the Na^{+} fluxes underlying the electrical phenomena. The experimental work is presented in the appendix, which consists of five papers. The first two chapters in the appendix deal with functional definitions of isolated rod outer segment preparations with respect to their capability to transport and store Ca^{2+} ions, their capability to perform specific interactions with Na^{+} and Ca^{2+} ions, as well as with respect to the structural integrity of the plasma membrane and the stacked pile of disks. The third chapter studies one important aspect of the proposed role of Ca^{2+} ions to act as intracellular transmitters: does light cause a rapid release of Ca^{2+} ions from rod outer segment disks? The last two chapters deal extensively with the properties of cation transport and storage in isolated rod outer segments. Correlates with electrophysiological observations on rod cells in the vertebrate retina are presented in the discussion sections of these two papers. In the following I will confine myself to a critical reviewing of the recent literature on the electrical responses of rod and cone cells and on the models, which have been developped to explain the transduction of an electromagnetic input signal into an electrochemical output signal. In chapter three an alternative proposal for the latter will be presented.

In the sixties two important new techniques were introduced to record electrical signals from photoreceptors in the retina in addition to the long-known transretinal potential, the so-called electroretinogram or ERG. In the first place, the progressive development of microelectrodes opened the way to record intracellularly from individual photoreceptor cells. Secondly, in the work of Penn, Hagins and Yoshikami (Penn and Hagins, 1969 and 1972, Hagins et al., 1970)

a voltage gradient along the length of rod cells in the rat retina was scanned with microelectrodes under visual control. For a review of the earlier work on the electrical activity of the vertebrate retina, the resolution in its components and the isolation of the receptor potential, i.e. the electrical response of the photoreceptor cells, see Tomita (1970). The discussion presented here intends to review the data accumulated since the introduction of these new techniques and their first application.

2. THE HYPOTHESIS OF A DIFFUSABLE TRANSMITTER

2.1. Development of the concept of a diffusable transmitter

At the end of the sixties it had been established that both rod and cone cells respond to light by a hyperpolarization of their membrane potential (Tomita, 1970). Furthermore, the receptor potential was shown to depend on the presence of extracellular Na^+ ions, and it was suggested that a gradient of Na^+ ions was required across the plasma membrane of the photoreceptor cell The latter point was illustrated by the observation that in the presence of an inhibitor of the metabolic ion pump (ouabain, which inhibits the Na-K pump) restoration of a Na^+ gradient across the plasma membrane was sufficient to generate a receptor potential (Sillman et al., 1969). The experiments of Penn and Hagins (1969) showed that in the dark a steady current flowed along the length of a rod cell, which was progressively reduced with increasing illumination and which was abolished by 10 mM KCN. This was explained by a steady inward membrane current in the outer segments balanced by an equal outward current in the other parts of the rod cell, and maintained by a metabolic ion pump. Combined, these data led Sillman et al. (1969) to the suggestion that the primary action of light is to reduce the permeability of the outer segment plasma membrane for Na^+ ions (e.g. reduce the current).

Intracellular recordings of turtle cones by Baylor and Fuortes (1970) established that the current-voltage characteristic of the cone membrane showed a steeper slope (e.g. a higher resistance) in the light as compared to darkness. The hyperpolarizing light response increased to a saturation level with increasing light intensity following a simple rectangular hyperbola, described by the equation : $v/V_m = I/(I+\sigma)$, where v is the peak voltage response, V_m is the maximal voltage response at saturating lights, I is the light intensity and σ is the light intensity at which a half-maximal voltage response is recorded. These observations led to the suggestion, that the light-induced conductance change arises from the presence of a limited number of ionic channels in the outer segment plasma membrane, which can be closed by an intermediate substance produced by light (Baylor and Fuortes, 1970). The term channel refers to discrete structures, which can carry an elementary current and which can be closed

independently from each other by an interaction with the intermediate substance. The term channel will be used throughout this discussion and does not intend to suggest a specific molecular mechanism underlying its action (e.g. a "pore' or "carrier' type of transport system).

Kinetic analysis of the rising phase of photoresponses covering a 10^5-fold range of light intensities and upto 50 ms after a flash of light is in accordance with the model in which the production of the intermediate substance or transmitter increases linearly with the light intensity in both rods (Penn and Hagins, 1972) and cones (Baylor et al., 1974a). In agreement with this, the amplitude of small responses in both rods and cones (less than 2 mV, V_m is usually 20-30 mV) is directly proportional to the amount of light absorbed and independent of the distribution of quanta in flashes or steps of light (Baylor and Hodgkin, 1973, Schwartz, 1975) At later times and for larger responses non-linearities occur. In addition to the non-linearities due to the limiting time constant of the cell membrane and from the limited number of channels to be closed (Penn and Hagins, 1972, Baylor and Hodgkin, 1973 and 1974) These non-linearities were suggested to arise from the metabolism of the transmitter molecules and could be quantitatively explained by the suggestion that inactivation of the transmitter molecules produced by light occurs by an autocatalytic process (Baylor and Hodgkin, 1974, Baylor et al , 1974b) This model provides the useful prediction, that changes of the steady state concentration of transmitter will affect the kinetics and the peak voltage of photoresponses in a similar way as observed during light and dark adaptation. In the course of this discussion I will frequenlty come back to the implications of this model

When one makes the assumption that in rods as well as in cones the primary action of light is to produce the same transmitter molecules, which block the same type of channels, the topology of rod outer segments known at that time implied that the transmitter molecules diffused in the cytosol in order to bridge the gap between the site of the photopigments (disk membrane) and the site of the ionic channels (plasma membrane), see Figs. 2 and 3.

From the above data and considerations the following simple model was constructed. In the dark, Na^+ ions flow into the outer segments of photoreceptors

through ionic channels in the plasma membrane Upon illumination transmitter molecules are released, which diffuse in the cytosol to the ionic channels in the plasma membrane, bind to these channels and thereby block the inward current of Na^+ ions. In the following sections of this chapter the experimental support for the two candidates, which have been proposed until now in the literature, will be analyzed. In the subsequent chapters data and arguments will be presented, which may unsettle this simple model, which has dominated the last decade.

2.2. Ca^{2+} ions as a candidate for the intracellular transmitter

The suggestion of a transmitter substance released by light was further elaborated by Hagins and Yoshikami (Hagins, 1972, Yoshikami and Hagins, 1973, Hagins and Yoshikami, 1974). The dark current along the rod cell was thought to arise from the fact that the outer segment is predominantly permeable to Na^+ ions, whereas the inner segment is predominantly permeable to K^+ ions and contains the metabolic ion pump necessary to maintain the ionic gradients Inhibition of the Na-K pump by ouabain resulted in a rapid decay of the dark current and the photoresponses, which could be slowed down by a steady background light. This indicates that the turnover of intracellular Na^+ and K^+ ions is slowed down by light, which suggests that photoreceptor cells hyperpolarize in the light due to a conductance decrease. Since the action of light is confined to the site of the photopigments, i.e. the outer segment, it was concluded, in agreement with the previous suggestion by Sillman et al. (1969), that light decreases the conductance of the outer segment plasma membrane for Na^+ ions [§] In their experiments Yoshikami and Hagins (Yoshikami and Hagins, 1973, Hagins and Yoshikami, 1974) further showed that an increase of the extracellular Ca^{2+} concentration mimicked the effect of light in decreasing the dark current and in slowing down the turnover of intracellular Na^+ and K^+ ions, whereas a decrease of the extracellular Ca^{2+} concentration increased the dark current. Their interpretation of these data was that changes of the extracellular Ca^{2+} concentration are followed by concomitant changes of the cytosol Ca^{2+} concentration in the

[§] *An increase in the conductance of the outer segment for K^+ ions would also result in a hyperpolarization of the plasma membrane and in a reduction of the dark current, but would lead to an increase of the turnover of intracellular Na^+ and K^+ ions.*

same direction (Ca^{2+} concentration means free Ca^{2+} concentration unless indicated otherwise) This would mean that raising the intracellular Ca^{2+} concentration decreases the dark current by blocking ionic channels, whereas lowering the intracellular Ca^{2+} concentration has the opposite effect. This led them to the suggestion that Ca^{2+} ions are identical with the transmitters produced by light (Fig. 4, taken from Hagins, 1972). In cones, light was thought to result in an influx of Ca^{2+} ions from the extracellular space down the concentration gradient across the plasma membrane into the cytosol. This would lead to an increase of the cytosol Ca^{2+} concentration, which in the dark is kept at a low value.

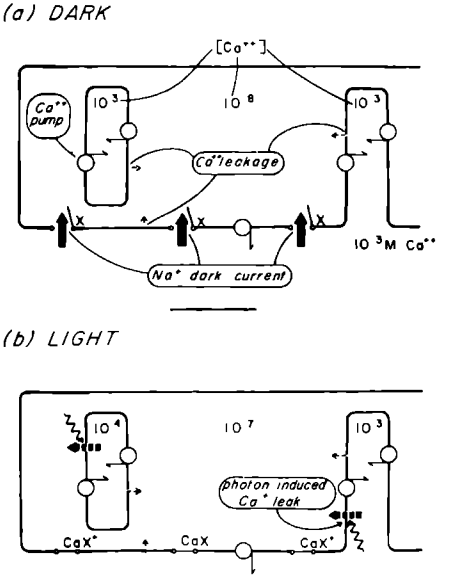

Fig. 4. Schematic presentation of the Ca^{2+} transmitter hypothesis. The left-hand side of the picture represents a rod outer segment and the right-hand side of the picture a cone outer segment. In later work Hagins and Yoshikami have estimated a Ca^{2+} concentration in the dark of about 1 μM in rat rods (Hagins and Yoshikami, 1975 and 1977). Taken from Hagins (1972).

By analogy, the disk interior in rods was thought to maintain a high Ca^{2+} concentration as compared to the cytosol. Light should cause an influx of Ca^{2+} ions from the disk interior down the concentration gradient into the cytosol In the

cytosol, Ca^{2+} ions diffuse to the Na^+ channels in the plasma membrane and block the inward Na^+ current. This model is illustrated by Fig. 4.

In support of these data, Brown and Pinto (1974) have shown by intracellular recording from individual rod cells in the toad retina that changing the extracellular Ca^{2+} concentration mimicks the effect of light on the membrane potential. Thus, increasing the extracellular Ca^{2+} concentration hyperpolarizes the membrane potential in the dark, but not in the light, whereas decreasing the extracellular Ca^{2+} concentration depolarizes the membrane potential in the dark, but not in the light. This means that the voltage responses upon illumination are decreased in the former and increased in the latter case of changing the extracellular Ca^{2+} concentration.

A next step was to manipulate the cytosol Ca^{2+} concentration directly. This has been achieved by Brown et al. (1977) by iontophoretic injections of Ca^{2+} and of EGTA (a chelator, which binds Ca^{2+} ions as opposed to Mg^{2+} ions at physiological pH). Injection of Ca^{2+} ions in toad rods hyperpolarizes the membrane potential. This effect decays in a similar way as the membrane hyperpolarization evoked by light. Injection of EGTA attenuates the responses to dim flashes. This effect decays with a half time of 20 s. In another approach, Hagins and Yoshikami (1977) introduced various Ca^{2+}-chelating substances into the rods of the rat retina by a vesicle fusion technique. When EGTA was introduced the authors found an attenuation of responses, but only to dim lights. The above experimental results are consistent with the suggestion that light causes an influx of Ca^{2+} ions into the cytosol, and that stronger lights mask the presence of EGTA in the cytosol by an appropriate Ca^{2+}-influx. In their experiment, Hagins and Yoshikami (1977) estimated the cytosol EGTA concentration to amount to 11 μM, and therefore stronger lights must result in a Ca^{2+}-influx of at least comparable magnitude. Hagins and Yoshikami (1977) have calculated on the basis of these experiments, that one photon results in a Ca^{2+}-influx of at least 400-900 Ca^{2+} ions

2.3. A critical commentary on the mechanism of the proposed light-induced increase of the cytosol Ca^{2+} concentration

When in the above described experiments of Hagins and Yoshikami (1977) EGTA was introduced into the cytosol of the rods in the rat retina, the attenuation of responses to dim flashes of light was stable even for two hours in the case

that an extracellular medium with a low Ca^{2+} concentration was used (pCa=6 7). On the other hand, upon raising the extracellular Ca^{2+} concentration to a pCa of 4 the responses at all light levels became indistinguishable from the control experiments where the vesicles did not contain EGTA. These observations suggest that in rods the control of the cytosol Ca^{2+} concentration (Ca^{2+}-homeostasis) is carried out by the plasma membrane, most likely by a Na-Ca exchange mechanism (Schnetkamp, 1980). It should be emphasized that this notion is implicit to the interpretation that changes of the extracellular Ca^{2+} concentration are almost instantaneously followed by concomitant changes of the cytosol Ca^{2+} concentration in the same direction (Hagins and Yoshikami, 1974; see also previous section 2.2). This, however, is difficult to reconcile with the original presentation of the Ca^{2+}-transmitter hypothesis (Fig. 4). The increase of the cytosol Ca^{2+} concentration upon illumination was thought to arise from a light-induced Ca^{2+}-release from disks. In the dark, the original Ca^{2+} concentration should then be restored by a Ca pump. This pump must be of considerable capacity and must be precisely fixed at the Ca^{2+} concentration prevalent in darkness, since it is capable to counteract within a few seconds the rise of the cytosol Ca^{2+} concentration upon saturating flashes of light. If Ca^{2+} ions are released from disks in the light they must be pumped back into the disks in the dark, since otherwise the rod cell would continuously lose Ca^{2+} ions upon repetitive stimulation with light. Thus, a Ca pump must be located in the disk membranes. However, a Ca pump in the disk membrane cannot discriminate between changes of the Ca^{2+} concentration in the cytosol caused by a light-induced release of Ca^{2+} ions from disks or by changes of the extracellular Ca^{2+} concentration. Therefore, rapid changes of the cytosol Ca^{2+} concentration upon changes of the extracellular Ca^{2+} concentration are incompatible with the presence of the proposed Ca pump in the disk membranes. If Ca^{2+}-homeostasis in the rod cytosol would be maintained by structures in the plasma membrane, a light-induced release of Ca^{2+} ions down a concentration gradient across the disk membrane as a mechanism for the increase of the cytosol Ca^{2+} concentration upon illumination seems impossible in view of the depletion argument mentioned before In support of this line of reasoning.

a- Hagins and Yoshikami (1974 and 1975) have observed that in the presence of ionophores for divalent cations (X537A and A23187), which would shortcircuit a hypothetical Ca pump in the disk membrane, photoresponses still can be

recorded provided that the extracellular Ca^{2+} concentration does not exceed 10 μM In view of the lipophilic character of these ionophores and of the close apposition of the plasma membrane and the disk membranes, the ionophores will distribute themselves over both membranes and will abolish all steady state Ca^{2+} gradients. These points are illustrated in this study for the case of A23187 and isolated intact rod outer segments (Schnetkamp, 1979) On the other hand, the experiments with ionophores do support the suggestion, that Ca^{2+}ions in the rod cytosol block light-regulated channels much more effective than Ca^{2+}ions in the extracellular medium.

b- Under conditions that a Ca^{2+} gradient across the disk membrane exists no release of Ca^{2+}ions with the required stoichiometry and within the time course of visual excitation could be demonstrated until now. An experimental approach to this problem and a discussion on the data published by other authors is offered in chapter III of the appendix (Kaupp et al., 1979).

c- An extensive investigation of the Ca^{2+} metabolism of isolated rod outer segments is presented in the last two chapters of the appendix (Schnetkamp, 1979 and 1980). It is shown that Ca^{2+}ions in rod outer segments are predominantly stored in disks and almost exclusively by binding. No large Ca^{2+} gradients across the disk membrane occur despite of the fact that isolated rod outer segments may contain considerable amounts of Ca^{2+}ions. Under conditions that a hypothetical Ca pump is shortcircuited (in the presence of the ionophore A23187) and would be forced to work continuously, no substantial depletion of metabolic energy, available in the cytosol of isolated rod outer segments, was found even on a time scale of a hour.

d- The light-induced influx of Ca^{2+}ions in cone cells is suggested to arise directly from the extracellular space (Fig. 4). If in rods and cones the Ca^{2+} concentration in the external medium is lowered it should directly affect the photoresponses in cones, but in rods smaller and delayed effects should be expected. In the latter case, the store of Ca^{2+}ions, from which Ca^{2+}ions are released upon illumination, is thought to be localized in disks (Fig.4) and shielded from the extracellular space by two membranes and a thin zone of the cytosol In contrast, the effects of Ca^{2+}-free media and the time course of these effects have been reported to be strikingly similar for cones (Bertrand et al., 1978, Arden and Low, 1978) and rods (Hagins and Yoshikami, 1974, Lipton et al., 1977a).

In conclusion, the above arguments seem to exclude the model, in which light causes a release of Ca^{2+} ions from rod disks. If light causes an influx of Ca^{2+} ions in the cytosol of rod and cone cells, any model should account for the observed similarities between rods and cones.

2.4. A modified proposal for the mechanism of a light-induced increase of the cytosol Ca^{2+} concentration in rod and cone cells

An alternative mechanism for a rapid light-induced increase of the cytosol Ca^{2+} concentration, which is in accordance with the observed similarity between rods and cones, may be suggested on the basis of the properties of the cation transport system present in rod outer segments and described in this investigation (Schnetkamp, 1979 and 1980):

a- This system forms a communication pathway between rod disks and the rod plasma membrane since it has access to both the intradiskal and the extracellular space, and possibly also to the cytosol compartment (Schnetkamp, upublished observations). Therefore, in this aspect the implications of the topological difference between rods and cones do not apply.

b- This system exists in at least two states and in one state it performs exchange transport of Na^+ and Ca^{2+} ions.

c- The exchange transport mode can be regulated by an "on-off" switch.

d- The relative affinities for Na^+ and Ca^{2+} ions are subject to control.

e- The unidirectional Ca^{2+}-flux may amount up to 6×10^6 Ca^{2+} ions/rod outer segment per s.

f- An affinity of 0.16 - 1 μM for Ca^{2+} ions theoretically allows Ca^{2+}-influxes from external media with Ca^{2+} concentrations as low as 0.1 μM.

With these properties in mind one could think of the following scheme. Photolysis of a rhodopsin molecule in the disk membrane is communicated to the transport unit residing in that disk. This information is transferred to the plasma membrane counter part of this transport system and might result in a switch over of the latter from a predominant electrogenic transport mode to a Na-Ca exchange transport resulting in a net influx of Ca^{2+} ions in the cytosol. If a cattle rod outer segment contains 500 disks and if each disk contains one transport unit (Schnetkamp, 1980) a maximal influx of 10^4 Ca^{2+} ions per second would be conceivable. In view of the observation that illumination produces only

a local effect (Hagins et al., 1970) the influx of Ca^{2+} ions will be confined to a zone of say 50 disks and in the absence of Ca^{2+}-buffers could maximally result in an increase of the cytosol Ca^{2+} concentration by 10 μM. In view of the affinity for Ca^{2+} ions it is interesting to note that for rods the Ca^{2+} concentration in the external medium has to exceed 0.2 μM in order to obtain stable photoresponses (Hagins and Yoshikami, 1977).

The elegancy of the original hypothesis of Hagins and Yoshikami lies in the unifying explanation of transduction in rods and cones Irrespective of the above modification of this hypothesis, in cones the light-induced Ca^{2+}-influx originates from the extracellular medium and consequently a complete removal of extracellular Ca^{2+} ions is expected to abolish light responses. This has been observed by Yoshikami and Hagins (1978) for the cones of the pure cone retina of the lizard *Iguana Iguana*, but is not observed by Bertrand et al (1978) for the cones of the turtle retina and by Arden and Low (1978) for the cones of the pigeon retina. In the latter case as well as for the rods of the rat retina (Hagins and Yoshikami, 1974) and for the rods of the toad retina (Lipton et al., 1977a) it is observed that prolonged exposure to Ca^{2+}-free media results in a progressive desensitization of the retina and finally after 10-15 minutes in an abolition of the responiveness to light. Hence, to maintain the Ca^{2+} transmitter hypothesis one must assume that in the direct vicinity of the outer segment plasma membrane Ca^{2+} concentrations of at least 0.1 μM are maintained for periods up to a few minutes after the start of the perfusion of the retina with a Ca^{2+}-free (EGTA containing) medium.

2.5. Further experimental tests for the role of Ca^{2+} ions as intracellular transmitter

An experimental test for the Ca^{2+} transmitter hypothesis could be carried out by the use of ionophores for Ca^{2+} ions It has been argued before that application of such ionophores will abolish steady state Ca^{2+} gradients. Therefore, if light causes a transient increase of the cytosol Ca^{2+} concentration, the kinetics of photoresponses should be altered by increasing ionophore concentrations or by the use of a more efficient ionophore (A23187). However, the use of A23187 will lead to the complication that as opposed to the situation without A23187 the binding capacity of the disk interior is made accessible also

to Mg^{2+} ions. This complication cannot be prevented by simple omission of Mg^{2+} ions in the external medium, because this in its turn will result in a complete inhibition of all processes in the cytosol, which use MgATP as a high-energy substrate (Schnetkamp, to be published).

A discriminatory test for every transmitter candidate has been suggested by Bertrand et al. (1978) on the basis of the kinetic model of Baylor et al. (Baylor and Hodgkin, 1974, Baylor et al., 1974a and b). In addition to the release of transmitter molecules light causes a desensitization, which is delayed with respect to the peak of the voltage response (Baylor and Hodgkin, 1974). In the above model this is quantitatively explained by the assumption that the transmitter molecules are removed by a series of reactions, the first of which is an autocatalytic step. This implies that the steady state concentration of transmitter molecules determines the rate of the inactivation reaction. A reduction of the steady state transmitter concentration (in the case of Ca^{2+} ions by a reduction of the external Ca^{2+} concentration) would result in larger responses, which last longer, and these effects should be counteracted by background lights. Conversely, an increase of the steady state transmitter concentration should, as observed for background lights, result in a voltage compression and in a shortening of the time scale of photoresponses. These tests have been carried out for the cones of the turtle retina by Bertrand et al. (1978) and it was found that, although light and Ca^{2+} had similar effects on the voltage response, Ca^{2+} ions, as opposed to background light, had little effect on the time scale of photoresponses. In agreement with this, Lipton et al. (1977a) have observed with toad rods that changes of the external Ca^{2+} concentration alter the amplitude of photoreponses, but have little or no effect on σ, the light intensity at which a half-maximal response is obtained.

It could be argued that the above tests only apply for the model in which attenuation and shortening of responses superimposed on background lights is caused by the autocatalytic inactivation of transitter molecules. Independent of the correctness of this model, it should be noted, that for cones it is observed that the sensitivity and the membrane potential recover with an identical time course, when the latter ranges between 0.5 and 40 s after adapting lights of different intensity (Baylor and Hodgkin, 1974, see Fig. 13). For rods it is observed that recovery of sensitivity lags somewhat behind the recovery of the membrane potential (Penn and Hagins, 1972, Kleinschmidt and Dowling, 1975,

Lipton et al., 1977a), but changes in the time course of the latter by variation of the medium conditions are always closely followed by parallel changes in the time course of the recovery of the sensitivity (Lipton et al., 1977a). These data are difficult to explain in another way than by the suggestion that desensitization by background lights is directly related to changes in the steady state concentration of transmitter molecules, and therefore these results strengthen the validity of the tests suggested by Bertrand et al. (1978).

If, nevertheless, the desensitization by background lights is caused by a process, which is independent of the concentration of transmitter molecules, changes of the steady state concentration of the latter should still affect the value of σ, although to a much lesser degree. On the assumption that transmitter molecules interact in a simple one to one manner with a limited number of channels, it can be calculated that, as compared to a situation where 90% of the channels are open, a situation where only 10% of the channels are open in the dark, should result in a shift of σ by one logunit (when the above percentages of channels open in the dark are 80% repectively 20%, the shift of σ should amount 0.6 logunit). Unfortunately, there is no agreement in the literature with respect to the shifts of σ upon changes of the extracellular Ca^{2+} concentration. Most authors do not find any shift of (Yoshikami and Hagins, 1973, Hagins and Yoshikami, 1977, Arden and Low, 1978). The argument of the first authors that after a two-minute exposure of a rat retina to a low Ca^{2+}-medium (pCa > 7) the value of σ in rods is increased by one logunit does not apply, because initially σ is not changed despite of the fact that the large increase of the dark current indicates that the steady state transmitter concentration has changed. In addition, desensitization normally develops within one second after the onset of a background light. On the other hand, in a recent report, Fleming and Brown (1979) do report shifts of σ upon changes of the extracellular Ca^{2+} concentration, which are consistent with the above calculation. Curiously, these authors draw exactly the opposite conclusion. They assume that Ca^{2+} ions are involved in light adaptation rather than play a role as transmitter molecules. However, the shift of σ observed by them is much to small and besides Ca^{2+} ions and light have different effects on the time scale of photoresponses, i.e. Ca^{2+} ions do not produce the characteristic shortening of photoresponses (Bertrand et al., 1978).

2.6. cGMP as a candidate for the intracellular transmitter

Although somewhat beyond the scope of this thesis, the possible role of guanosine 3', 5'-cyclic monophosphate (cGMP) is discussed here, because at present it is the only serious alternative to Ca^{2+} ions, which has been proposed in the literature. In recent years the importance of cGMP in photoreceptor functioning has become increasingly clear This interest was initiated by the discovery by the group of Bitensky that bleaching of rhodopsin molecules stimulates a phosphodiesterase (enzyme, which catabolizes cGMP),endogenous to rod outer segments, in a process, which saturates at low bleaching levels (for a review, see Bitensky et al., 1978) Here, the discussion will be confined to a few recent papers in the perspective that cGMP is a possible candidate for the intracellular transmitter in rod cells A more extensive review is given by Hubbell and Bownds (1979).

Liebman and Pugh Jr. (1979) calculate that under optimal conditions stimulation of the rod outer segment phosphodiesterase by light results in an amplification, which would allow a sufficient depletion of cGMP levels in the rod cytosol upon the bleaching of only one rhodopsin molecule/outer segment and within the time domain of visual excitation The authors present a model in which the negative transmitter (i e. a transmitter, whose concentration is reduced by light) cGMP affects the phosphorylation and dephosphorylation of the light-regulated channels. In agreement with this result, Woodruff and Bownds (1979) report that light causes a rapid (half time $\lesssim$ 125 ms) decrease of the cGMP content of isolated frog rod outer segments. Extrapolation of the experimental data to the bleaching level of one photon/outer segment suggests that the latter could result in a 3% decrease of the cytosol cGMP level. However, the strong non-linearity observed between the amount of rhodopsin molecules bleached and the number of cGMP molecules hydrolized is in conflict with the kinetic analysis of the rising phase of photoresponses, which infers that the depletion of a negative transmitter increases linearly with the absorbed photons (Penn and Hagins, 1972, Baylor et al., 1974a). Furthermore, similar experiments, carried out with intact frog retinae, fail to detect this rapid decrease of cGMP levels upon illumination (Kilbride and Ebrey, 1979). These authors find a slower (several seconds) decrease of cGMP levels in the frog retina and only at illumination levels, which bleach a few hundred rhodopsin molecules per outer segment.

The cGMP content of frog rod outer segments amounts to 3-6 x 10^7 cGMP molecules/outer segment (Woodruff and Bownds, 1979, Kilbride and Ebrey, 1979), whereas a frog rod outer segment contains 3 x 10^9 rhodopsin molecules. More than half of this cGMP pool is not affected even by the strongest lights, which bleach all the rhodopsin present (Cohen et al., 1978, Woodruff and Bownds, 1979, Kilbride and Ebrey, 1979). In addition, the first authors have reported that incubation of mouse retinae in a Ca^{2+}-free medium (pCa=9), but not in media with a pCa between 4 and 5, results in a transient increase of the cGMP content by tenfold. Under these conditions, the drop in the cGMP content by illumination may be up to fifteenfold. Woodruff and Bownds (1979) have performed most of their experiments with isolated frog rod outer segments in a Ca^{2+}-free medium, but have not observed this effect.

Agents, which are thought to increase the cytosol cGMP concentration, produce similar effects on the photoresponses of toad rods as were previously noted for Ca^{2+}-free media (Lipton et al., 1977b). The membrane potential is depolarized, the voltage response on illumination is increased, but the value of σ is hardly or not affected. These results are qualitatively consistent with the results of Cohen et al. (1978) mentioned above. However, quantitative agreement is less convincing. Lowering the pCa in the external medium to 4 or 5 has no effect on the cGMP content of the mouse retina (Cohen et al., 1978), but has strong effects on the membrane potential, voltage response and dark current of rods (Hagins and Yoshikami, 1974 and 1975, Brown and Pinto, 1974, Flaming and Brown, 1979). Also, the effect of the phosphodiesterase inhibitor IBMX on the cGMP content of the mouse retina is rather modest as compared to the effect of a Ca^{2+}-free medium (Cohen et al., 1978), but the effect of IBMX on the membrane voltage and light-induced voltage response of toad rods is rather pronounced (Lipton et al., 1977b).

Agents, which are thought to increase the intracellular concentration of cGMP shorten the photoresponse (Lipton et al., 1977b). Following the suggestions of Bertrand et al. (1978) the kinetic tests discussed in section 2.5 predict that a reduction of the steady state transmitter concentration should slow down the time scale of photoresponses An increased concentration of the negative transmitter cGMP corresponds to a reduced steady state concentration of blocked channels and should according to the autocatalytic model slow down the responses. The experimental data show the opposite behaviour (Lipton et al., 1977b).

If one makes the assumption that low Ca^{2+} media affect the photoresponses only indirectly by increasing the intracellular concentration of cGMP, a tenfold increase in the concentration of the hypothetical transmitter cGMP in low Ca^{2+} media is bound to affect the kinetics and sensitivity of photoresponses. However, as discussed in section 2.5 these effects are not observed.

Intracellular injection of cGMP into the rods of the toad retina results in a transient depolarization of the membrane potential approximately to the Na^+ equilibrium potential and in a delay of the responses to light (Nicol and Miller, 1978, Miller and Nicol, 1979). From this the authors suggest that an increased cytosol concentration of cGMP has to be degraded. However, this suggestion is not in agreement with the current-voltage characteristics of rods, which show that a hypothetical light-regulated conductance is overshadowed by light-insensitive conductances, associated with K^+ ions (see section 4.3). Therefore, an agent, which causes a depolarization of the membrane potential to the Na^+ equilibrium potential, would more likely interfere with K^+ channels rather than with Na^+ channels. With regard to the second part of their interpretation, the increase of the phosphodiesterase activity by light is so large (Bitensky et al., 1978, Yee and Liebman, 1978) that the recovery from a cGMP injection in the dark (Miller and Nicol, 1979) is much to fast as compared to the delays of photoresponses.

In conclusion, the stimulation of the rod outer segment phosphodiesterase by low light levels and the high activity of this enzyme are interesting properties with regard to the option that cGMP is a candidate for the intracellular transmitter in rod cells. On the other hand, the large light-insensitive cGMP pool in rod outer segments and the fact that strong variations of the intracellular cGMP concentration have no clear effects on the time scale of photoresponses and on the sensitivity of the rod cell raise fundamental objections. It should be added, that the experimental data of the various authors contain a number of discrepancies, which are not always adequatly discussed and therefore confuse a clear picture.

3. AN ALTERNATIVE TO THE CONCEPT OF A DIFFUSABLE TRANSMITTER IN THE CYTOSOL

3.1. Prelude

From the preceding chapter one may draw the conclusion that fundamental objections against both of the candidates for the diffusable intracellular transmitter arise from the fact that changes of the steady state concentration of both of these candidates lack to give clear effects on the time scale and sensitivity of photoresponses. Therefore, it may be useful to explore another possibility. As introduction, a curious difference in the morphology of rod outer segments of different vertebrate species will be discussed.

Rod outer segments of different species have rather diverging dimensions. Although no exact data are published, cattle rod outer segments are cylinders with dimensions of about 1 x 20 μm (diameter x length), whereas mudpuppy rod outer segments are cylinders with dimensions of about 12 x 60 μm This means that the volume of a mudpuppy rod outer segment is about 430 times larger than that of a cattle rod outer segment. If one makes the assumption that all vertebrate species use the same transduction machinery and the same transmitter substance, it will be clear that on the absorption of a single photon per outer segment the production or depletion of a diffusable transmitter in the cytosol must be at least 430 times larger for a mudpuppy rod outer segment than for a cattle rod outer segment. Moreover, the steady state transmitter concentration in darkness and its rise in the cytosol upon absorption of a single photon are most likely very similar in both species in view of the similar values of σ reported for the large mudpuppy rod cell (31 photons absorbed/outer segment, Fain and Dowling, 1973) and for the small rat rod cell (30-50, Penn and Hagins, 1972). However, there are no reports on the biochemistry of rod outer segments, which indicate the presence of an enzymatic machinery which is capable to compensate for the volume difference. For instance, the phosphodiesterase activity is reported to be very similar in the large frog rod outer segments as compared to the small cattle rod outer segments (Yee and Liebman, 1978). In summary, the large variation in volume between rod outer segments of different species has serious consequences, if one assumes that visual transduction is carried out by a diffusable transmitter in the

cytosol. On the other hand, it is interesting to report the observations by Papermaster et al. (1978), which suggest a topological rather than a chemical adaptation to the increase of size of the outer segment At increasing size of the rod outer segment the disks are divided by incisures in such a way that an individual rhodopsin molecule is never >0.5 μm from the nearest internal or marginal disk edge. This seems to suggest that diffusion of rhodopsin molecules to a site in the disk edge may be relevant for the functioning of visual excitation.

3.2. Arguments against the presence of a diffusable transmitter in the cytosol

It has recently been shown that in the large toad rods (diameter of the outer segment about 8 μm) quantal events invariably evoke responses, which are uniform in amplitude and kinetics (Baylor et al., 1979b). From this and from thermodynamic arguments (heat of activation of the rise time of responses, Baylor et al., 1974a) the authors conclude that diffusion of transmitter molecules, released by a photoisomerization at any given position in the disk membrane, to the channels in the plasma membrane cannot be the major constituent in the time course of a photoresponse. This will apply for both radial and axial diffusion in the outer segment. In the latter case, photoisomerization of a rhodopsin molecule located near the cleft between the plasma membrane and the stacked pile of disks would be expected to give rise to a greater axial spread. However, Hagins et al. (1970) and Jagger (1979a and b) have observed that local illumination of a zone of the outer segment results in a response, which is confined to a limited spread in the length axis of a rod outer segment. These results suggest that diffusion of transmitter molecules in a rod cell causes only a limited spread in the length axis and must therefore be a major determinant in the time course of a photoresponse. These arguments together seem to exclude a diffusable transmitter and were presented in the discussion of chapter V of the appendix (Schnetkamp, 1980). In the following the line of reasoning against a diffusable transmitter will be reinforeced.

In the most striking case of local effects on local illumination, Jagger (1979b, see Fig. 4) has observed that a strong flash of light bleaching 10^5 times more rhodopsin molecules than necessary for a threshold response and delivered at the one end of an isolated frog rod outer segment, desensitizes that part of the outer segment for at least one minute, but has no effect up to 36 s after the flash on the photoresponses recorded at the other end of that parti-

cular frog rod outer segment. The rise times of photoresponses of both rod and cone cells infer that over a 10^5 fold range of light intensities the concentration of transmitter molecules increases linearly with the light intensity (Penn and Hagins, 1972, Baylor et al., 1974a). In addition, the shape and amplitude of quantal responses recorded from electrically isolated rod outer segments (Yau et al., 1977, Baylor et al., 1979b) and the voltage responses of electrically coupled cells to single quanta of light (Fain, 1975, Schwartz, 1977) imply that transmitter molecules released upon the absorption of a single photon cover an area containing about 5% of the total amount of available ionic channels. This would mean that these transmitter molecules, which originate from a single disk, diffuse in the length axis of a rod outer segment over an area of about 50 disks. It is difficult to envisage that with light flashes, which produce 10^5 times more transmitter molecules (see above), a photoresponse would still be confined to a local spread in the length axis of a rod outer segment, certainly when one bears in mind that the major virtue and gain of the model of a diffusable transmitter lies in the ability , that transmitter molecules, released from a single disk, by diffusion may cover an area of channels, which substantially expands beyond that single disk. In conclusion, the observations underlying the above line of reasoning are difficult if not impossible to reconcile with the concept of a diffusable transmitter in the cytosol communicating between the bleaching of a rhodopsin molecule in the disk membrane and the subsequent conductance change of ionic channels in the plasma membrane.

From the previous paragraph it will be clear that every diffusable transmitter must sense a strong hindrance for diffusion in the length axis of a rod outer segment. However, such a proposal is bound to certain limitations. The effective diffusion coefficient for electrolytes in the rod cytosol is proportional to the conductivity of the rod cytosol. The latter has been determined in frog rod outer segments by Falk and Fatt (1973). These authors found a value of about 2% of the conductivity of a normal Ringer solution, which can be accounted for by the obstruction through the pile of stacked disks and which does not seem to allow effective compartimentation of the rod cytosol in the time domain of seconds (see appendix on diffusion times of Robinson, 1975). In addition, substantial lower values for the diffusion coefficient of small ions would lead to an electrical resistance in the outer segment cytosol of the same order of magnitude as observed for the plasma membrane. The passage of the dark current of Na^+ ions

through the rod cytosol would then generate a potential across the cytosol of comparable magnitude as observed for the membrane potential, which would lead to different membrane potentials in the inner segment and in the outer segment respectively. However, such differences are not observed (Bader et al., 1978, Werblin, 1978). One can now make the assumption that the rod cytosol contains a selective matrix, which allows free diffusion of Na^+ and Cl^-ions, but specifically adsorbs the transmitter. For example, a specific reduction of the effective diffusion coefficient for Ca^{2+}ions can be thought to arise from the presence of intracellular Ca^{2+}-buffering systems like mitochondria and sarcoplasmic reticulum. On the other hand, selective adsorption of the transmitter molecules will at the same time reduce the effectivity of the transmitter, because changes in its concentration will be levelled off by the this buffering system. In this context it may be useful to point to the phenomenal burden imposed on the metabolism of a rod cell by the fact, that the production or depletion of a transmitter increases linearly with the light intensity over 5 decades of the latter (see previous discussion). This means that the metabolic machinery has to deal with transmitter concentrations ranging from say 1 μM up to 100 mM, and this in addition to those substantial amounts of transmitter bound to the above buffering system.

3.3. An alternative to the concept of a diffusable transmitter in the cytosol

Notwithstanding the arguments put forward in the preceding section it may turn out to be possible to construct a model, which meets the objections. On the other hand, one could make the assumption that transduction is performed within the disk membranes themselves. Thus, the bleaching of a photopigment molecule causes a local change in the disk membrane, which via a multi-step delay and within the disk membrane is communicated to the site of the ionic channel and results in an interaction with the latter. In cones, the ionic channels and the photopigment molecules are embedded within the same membrane and as a first approximation one could suggest that the range of one photopigment molecule is confined to the disk, where it is located. Following the suggestion put forward in this investigation, that each rod disk contains one channel (Schnetkamp, 1980), one channel for every cone disk would then mean that absorption of one photon could result in the closure of (or more generally the interaction with) only one channel. Not inconsistent with the latter are the observations of Lamb and Simon (1977) and of Baylor et al. (1973 and 1974a). From noise analysis the first authors have estimated that in turtle cones

an elementary voltage event in the dark amount to 124 μV, and that additional noise in steady light corresponds to events of 110 μV. Following the suggestion of the authors that the latter result from photon events, since they have about the same size as the dark events, means that one photon closes one channel. Using the same preparation (turtle cones) Baylor et al. (Baylor and Hodgkin, 1973, Baylor et al., 1974a) have found that absorption of a single light quantum per outer segment gives a voltage response of 25 μV and closes 1/600 of the total number of channels. These authors estimate that a turtle cone has 800 disks, which would mean that each disk contains one channel on the assumption that one photon closes one channel.

If one suggests an analogous scheme as above for cones to work also for rods, two problems are obvious. Firstly, the topology of rod outer segments, i.e. the separation between photopigments and ionic channels in the disk membranes and the plasma membrane respectively,seems to contradict such a scheme. Secondly, a half-maximal voltage response is obtained at 30-100 photons absorbed/outer segment (Penn and Hagins, 1972, Fain and Dowling, 1973, Fain, 1975, Cervetto et al., 1977, Flaming and Brown, 1979), which seems to imply that one photon absorbed affects much more than one channel. Experimental observations, which may provide answers to these problems, are reported and discussed in this investigation (Schnetkamp, 1980). In brief, a transport system for Na^+ and Ca^{2+}ions is shown to constitute a direct connection between the rod plasma membrane and each rod disk. Furthermore, transport units in individual disks act interdependently by a non-stochastic process. This means, that theoretically from each individual disk an information transfer is possible, which includes a certain number of transport units, which have access to the extracellular space and which cover an area of more than one disk. One could speculate that the plasma membrane in rods as compared to the topology of cones functions as a shortcircuit communication pathway between individual disks.

At this stage it is interesting to recall the observations that in both rods and cones Ca^{2+}ions may greatly affect the dark current and the conductance of the ionic channels underlying this current (Yoshikami and Hagins, 1973, Brown and Pinto, 1974, Lipton et al., 1977a, Bertrand et al., 1978, Arden and Low, 1978, Flaming and Brown, 1979, see also sections 2.2 and 2.3), although the value for the light intensity at which a half-maximal response is recorded is not affected. This suggest that Ca^{2+}-induced changes in the conductance of

the ionic channels do not superimpose on the action of light, which means that every channel has the same chance to be closed by light irrespective of the fact that the average time individual channels are closed by Ca^{2+} ions may vary greatly This can be understood if Ca^{2+}-induced conductance changes of individual channels fluctuate rapidly as compared to the time scale of the photoresponses, whereas the light-induced conductance change of individual channelsmay result in a semi-permanent state, which does not fluctuate rapidly In other words, Ca^{2+} ions and light have a mechanistically different action From this behaviour an interesting analogy may be drawn to the properties of the cation transport system described in isolated rod outer segments (Schnetkamp, 1980). Na^{+} ions, which are thought to carry the dark current, act on two fundamentally different functions of this system. Firstly, they induce the above mentioned non-stochastic interaction between individual transport units, which results in a semi-permanent distribution of the transport states over the individual transport units. Secondly, Na^{+} ions compete in a stochastic manner with Ca^{2+} ions for a common transport site. On the basis of the observed affinities of this site for Na^{+} and Ca^{2+} ions and on the assumption that these ions show only ionic interactions with this site the fluctuations in the occupation by cations of individual sites will be very rapid ($<$1 ms)

From the above arguments and from the properties of this cation transport system a picture emerges of a transport system with multiple functions and different transport modes rather than a simple channel with a plug to close it One could suggest that the action of light is to switch over rather than to switch off a predominant electrogenic transport mode to a predominant exchange transport mode. The advantage of asymmetrical exchange transport (Na-Ca) could be that the ion content of the intracellular compartments is rapidly changed without giving rise to potential changes The advantage of symmetrical exchange transport (Ca-Ca) could lie in an overflow function, which does not draw upon the energy supply. For the ion fluxes in rod outer segments the latter is most probably the electrochemical Na^{+} gradient maintained by a metabolic ion pump in the inner segment

4.1. A simple electrical equivalent circuit for the cone membrane

Although this investigation was confined to experiments on rod outer segments, it may be useful to discuss first a simple electrical equivalent circuit for cones presented by Baylor et al. (1974a) and depicted in Fig. 5 The cone membrane appears to present a somewhat simpler case than the rod plasma membrane.

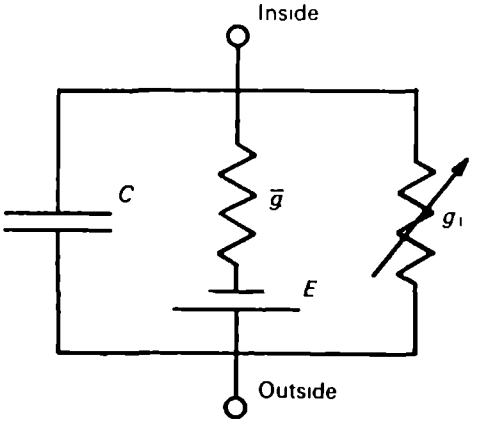

Fig. 5. Electrical equivalent circuit for the cone membrane, taken from Baylor et al. (1974a). C = the membrane capacitance, $\bar{g}$ = a fixed resistance in series with the battery E, and g_1 = a variable conductance reduced by light.

If the variable conductance g_1 is reduced to zero by saturating light or when the ions flowing through this conductance are removed, the membrane potential should become equal to E. E is the equilibrium potential for the ions flowing through the fixed conductance $\bar{g}$. In accordance with this simple model, Cervetto (1973) has observed that upon substitution of Na^+ ions in the extracellular medium by choline the membrane potential hyperpolarized to the same value, which was reached at the peak of a saturating photoresponse. This suggests that the ions flowing through the variable conductance g_1 can be identified as Na^+ ions. Increasing the extracellular K^+ concentration depolarized the membrane potential

in the dark, but much more in the light This is consistent with the notion that E represents the equilibrium potential for K^+ ions, which flow through the fixed conductance $\bar{g}$.

The resistance of the above described conductances can now be determined by injecting current in the cone cell and measuring the voltage change either in the light or in darkness. For a series of current intensities the relation between the injected current and the concomitant voltage changes (the so-called current-voltage characteristic) is shown to be linear both in the dark and in the light (Baylor and Fuortes, 1970, Lasansky and Marchiafava, 1974) This indicates that at least within the physiological range of membrane potentials both g_1 and $\bar{g}$ behave approximately according to Ohm's law The dark and the light current-voltage curves intersect and the photoresponse reverses sign at a membrane potential between +20 and +30 mV, which means that the equilibrium potential for the ions flowing through the variable conductance g_1 (most likely Na^+ ions) lies between +20 and +30 mV Although the values of the resistance of the cone membrane in the light and in darkness reported in the above papers differ by a factor of four, the ratio $g_1/\bar{g}$ is rather similar and amounted to 0.32 and 0.34 repectively. The scatter in the absolute values for the resistance of the cone membrane is due to a variable degree of electrical coupling between individual cone cells (Baylor and Hodgkin, 1973, Baylor et al , 1974a, Lamb and Simon, 1976) Taking the cone cell with the highest resistance from Baylor et al (1974a, cone no. 2 of table 2) to be an isolated cone, the resistance of the parallel connection of g_1 and $\bar{g}$, i.e. the membrane resistance in darkness, was 173 Mohm. In the light, the membrane resistance is equal to $\bar{g}$ and amounted 226 Mohm. From this it follows that g_1 amounted to about 740 Mohm in the dark. For that individual cone the membrane potential in the dark was -37 mV and in the light -48 mV. Dark membrane voltages of cones cited in the literature range between -25 and -45 mV, and membrane voltages in the light between -40 and -65 mV.

When the equilibrium potential for the ions flowing through the variable conductance g_1 is taken to be +25 mV (Baylor and Fuortes, 1970, Lasansky and Marchiafava, 1974), the driving potential for these ions in the above cited cone was -62 mV. Across a resistance of 740 Mohm this driving potential would lead to a current of 84 pA This value compares reasonably well with the values, which are reported for rod cells (20-70 pA in rat rods, Hagins and Yoshikami, 1975, and

6-27 pA in toad rods, Baylor et al., 1979a). It has been discussed before (section 3.3) that a cone may contain 600-800 light-regulated channels and that one photon closes one channel. If this were true an elementary current event would amount 0.12 pA and an elementary conductance change would be about 2.0 pS.

Lowering of the extracellular Ca^{2+} concentration depolarizes the membrane of cones in the dark, but does not affect the membrane voltage in strong lights (Bertrand et al., 1978) Calculation of their data learns that the ratio $g_1/\bar{g}$ was increased from 0.88 in a normal Ca^{2+}-medium to 3.27 in a low Ca^{2+}-medium (pCa= 9). It is suggested by the authors that this change is due to an increase of g_1 rather than a decrease of $\bar{g}$. In support of these data, Arden and Low (1978) report that the dark current of pigeon cones increased upto seven-fold when the pCa in the extracellular medium was increased to 9, although in their interpretation both g_1 and $\bar{g}$ increase.

In the foregoing it is suggested that a relatively simple model for the electrical properties of the cone membrane can account for most of the observations. In addition to the conductances of the equivalent circuit described above, Baylor et al. (1974b) have introduced a voltage-dependent conductance in order to account for the sag of the peak hyperpolarization to a plateau value in strong lights. In view of the linear current-voltage characteristic (Baylor and Fuortes, 1970, Lasansky and Marchiafava, 1974) and because this effect is not always so pronounced (Lasansky and Marchiafava, 1974, Bertrand et al , 1978) this complication has been omitted here for the sake of clarity.

4.2. Similarities in the electrical properties of rod and cone cells

The electrical responses of rod cells are remarkably similar to those of cone cells:

a- In the dark, the membrane potential is kept at a more positive value than the K^+ equilibrium potential, and in the light the membrane is hyperpolarized (see Tomita, 1970).

b- Upon removal of extracellular Na^+ions the membrane hyperpolarizes (Cervetto, 1973, Brown and Pinto, 1974) and the current flowing along the photoreceptor cell in the interstitial space is abolished (Hagins and Yoshikami, 1975)

c- Reduction of the extracellular Ca^{2+} concentration results in a depolarization of the membrane in the dark, but not in the light (Brown and Pinto, 1974, Lipton et al., 1977a, Bertrand et al., 1978, Fleming and Brown, 1979), whereas the dark current along the photoreceptor cell is increased (Hagins and Yoshikami, 1974, Arden and Low, 1978).

d- An increase of the extracellular Ca^{2+} concentration above the normal value hyperpolarizes the membrane potential in the dark, but not in the light (Brown and Pinto, 1974, Lipton et al., 1977a, Bertrand et al., 1978, Fleming and Brown, 1979), whereas the dark current is reduced (Yoshikami and Hagins, 1973).

e- Increasing the extracellular K^{+} concentration depolarizes the membrane, but much more effectively in the light than in darkness (Cervetto, 1973, Brown and Pinto, 1974).

The functional difference between rods and cones (see section 1.1) is electrically expressed by the different spectral sensitivities (see Tomita, 1970, Baylor and Hodgkin, 1973, Fain, 1975a), by the time scale and by the absolute sensitivity. Thus, rods saturate at lower light intensities as compared to cones, whereas the latter show a faster kinetics (both rising and falling phase). For a comparison of rod and cone responses recorded in the same retina see Baylor and Hodgkin (1973 and 1974), Fain and Dowling (1973) and Lasansky and Marchiafava (1974). For dark adapted rods the half-maximal voltage reponse is obtained at 30-100 photons absorbed/outer segment (Penn and Hagins, 1972, Fain and Dowling, 1973, Fain, 1975, Cervetto et al , 1977, Brown and Fleming, 1979) and for the most sensitive rods responses of 676 μV/absorbed photon/outer segment (Fain, 1975) and even 1175 μV/absorbed photon/outer segment (Brown and Fleming, 1979) are reported. For dark adapted cones half-maximal responses are obtained at 275-3500 photons absorbed/outer segment and values of 25-45 μV/absorbed photon/outer segment are reported (see Fain and Dowling, 1973).

4.3. Differences between the electrical properties of rod and cone cells

On the basis of the substantial similarities in the electrical behaviour of rod and cone cells summarized above it seems obvious to conclude that the simple model, which can account for the properties of cones, will also apply to rods, i.e. light closes channels for Na^{+} ions and this results in a hyper-

polarization of the membrane to the K^+ equilibrium potential. However. the latter clearly does not occur in rods. Saturating lights cause the rod plasma membrane to hyperpolarize to a value, which is considerably more positive than the membrane potential in the absence of extracellular Na^+ions (Brown and Pinto, 1974, Fain et al., 1978). The difference between rods and cones in this respect becomes particularly prominent when the dark potential is displaced by current injections and subsequent light responses are recorded. Injection of hyperpolarizing current in cones results in an increase of the voltage response to light, whereas injection of depolarizing current has the opposite effect (Baylor and Fuortes, 1970, Lasansky and Marchiafava, 1974). This can be understood by the notion that a hyperpolarizing current will increase the driving potential for Na^+ions flowing through the light-regulated conductance and a depolarizing current will reduce this driving potential (i.e. the difference between the Na^+ equilibrium potential and the prevalent membrane potential). In contrast, injection of hyperpolarizing current in rods results in a decrease of the voltage response to light and injection of depolarizing current initially (not to large current intensities) increases photoresponses (Lasansky and Marchiafava, 1974, Werblin, 1975 and 1978, Cervetto et al., 1977). This seems to suggest that light affects (in view of the hyperpolarizing response opens) channels for ions with an equilibrium potential more negative than the membrane potential. For example, if light would open K^+ channels in the outer segment this would cause a hyperpolarization of the membrane and also a reduction of the dark current in agreement with observation. The latter is caused by the fact that the spatial separation of Na^+ and K^+ channels, from which the dark current originates, would be partly abolished. The current-voltage characteristics in the dark and in the light give no clear answer to the question whether light causes a conductance increase or decrease. Instead these curves run parallel to each other, only shifted along the current axis but without intersection (Lasansky and Marchiafava, 1974, Werblin, 1978, but see Cervetto et al., 1977). Curiously, the photoresponse does reverse at a potential between 0 and +10 mV (Werblin, 1975 and 1978, Bader et al., 1978), which would be in reasonable agreement with a Na^+ equilibrium potential.

A possible clarification of this confusing situation may be offered by the work of Fain et al. (1978). Strong lights hyperpolarize the rod plasma membrane

showing a fast transient overshoot and a subsequent sag to a plateau value, which is considerably more positive than the K^+ equilibrium potential (see also Brown and Pinto, 1974, Kleinschmidt and Dowling, 1975, Lipton et al., 1977a, Cervetto et al., 1977). However, in the presence of 2 mM Cs^+ in the extracellular medium light causes the membrane potential to hyperpolarize to a value very near the K^+ equilibrium potential and without the transient overshoot (Fain et al., 1978). In darkness the membrane potential is not affected by the presence of 2 mM Cs^+ The current-voltage characteristic of rods is found to be steeper for hyperpolarizing currents than for depolarizing currents (Fain et al., 1978, Werblin, 1978 and 1979) Cs^+ions do not affect the current-voltage characteristic for depolarizing currents, but increase the steepness (increase the resistance) for hyperpolarizing currents. From these observations Fain et al (1978) propose that membrane hyperpolarization caused by the light-induced closure of Na^+ channels opens a voltage-dependent channel, which carries a Cs^+-sensitive current of Na^+ions. A remarkable aspect of the action of Cs^+ is the effectivity at such low concentrations. A similar sensitivity for Cs^+ions has been reported for the anomalous rectification of K^+ currents (Hagiwara et al., 1976). This might suggest that the rod channel also carries K^+ions, and with the previous argument that the equilibrium potential of such a channel should lie between the dark membrane potential and the K^+ equilibrium potential, these observations could suggest the presence of a voltage-dependent channel of low selectivity like the acetylcholine-sensitive channel. The inward current is predominantly carried by Na^+ ions and the outward current by K^+ions. Cs^+ions block only the current from the compartment where it is applied. This is a common phenomenon, when the blocking ion is to large to pass the channel, but does bind to the channel mouth. The equilibrium potential of such a channel would lie between the equilibrium potential for Na^+ ions (0 - +10 mV, Werblin, 1975 and 1978, Bader et al., 1978) and that for K^+ions (-80 mV, Fain et al., 1978). In agreement with this, the plateau hyperpolarization of rods with saturating lights is generally found to range between -30 and -50 mV (Fain and Dowling, 1973, Lasansky and Marchiafava, 1974, Cervetto et al., 1977, Fain et al., 1978)

The presence of a channel with the above properties could explain the observation that light causes rather variable changes of the rod resistance (Fain et al., 1978) and that these changes have the receptive field of a rod network

and not that of individual rod cells (Werblin, 1978). The latter means that the voltage-dependent properties of the rod plasma membrane and not the release of transmitter molecules in the rod cell, which is illuminated, predominantly determine the resistance changes observed upon illumination. In the presence of extracellular Cs^+ ions saturating lights cause an average increase of the resistance by 86 Mohm in toad rods (Fain et al , 1978). It could now be suggested that, similar as described previously for cones, this resistance increase arises from the light-induced closure of Na^+ channels However, the current-voltage characteristic for toad rods reveals that as compared to a depolarizing step a hyperpolarizing step in the dark and in the presence of 2 mM Cs^+ results in a resistance increase of 77.5 Mohm (calculated from Fig 3 of Fain et al., 1978) This means that a resistance increase accompanying a membrane hyperpolarization could result from the voltage-dependent properties of the rod plasma membrane and not necessarily from the light-induced release of transmitter molecules, which block ionic channels. Moreover, the lack of intersection of the current-voltage characteristics in the dark and in the light for depolarizing currents remains to be explained. If this were due to the fact that voltage-dependent channels also mask light-induced conductance changes at depolarized membrane potentials the question arises at which membrane voltage these channels are activated and for which ions, since the Cs^+-sensitive channel is thought to be activated at a membrane potential more negative than the dark membrane potential. In conclusion, at the present there is no satisfactory experimental evidence that the action of light in rod cells is to produce transmitter molecules, which block Na^+channels.

Electrical coupling between individual rod cells in the retina makes it difficult to assess the electrical resistance of a single rod cell within such a network (Schwartz, 1975 and 1976, Werblin, 1978, Detweiler et al., 1978) In two recent reports the current-voltage characteristic of isolated rod cells was recorded. Bader et al. (1978) use enzymatically isolated rods and find a S-shaped current-voltage characteristic with slope resistances between 240 and 1080 Mohm For membrane potentials between 0 and -20 mV (dark membrane potentials ranged between -36 and -54 mV without current injection) the current-voltage characteristic was linear and the resistances found in the dark and in the light were 530 and 680 Mohm respectively From this a light-induced decrease of the resistance by 2400 Mohm can be calculated Werblin (1979) uses mechanically isolated rods and the current-voltage characteristic obtained resembles

those reported for rod cells in the retina (Fain et al., 1978, Werblin, 1978) showing slope resistances between 10 and 100 Mohm These value are more than one order of magnitude lower than those of Bader et al. (1978).

Using a preparation (toad rods), of which the outer segments have about the same (large) dimensions as the above preparations of isolated rod cells, Baylor et al. (1979a) have reported that the dark current of electrically isolated rod cells, recorded at the outer segment, ranges between 6 and 27 pA This dark current is completely abolished by strong lights. When one assumes that the driving potential for Na^+ ions is 40 mV and that Na^+ ions flow through light-regulated channels, the light-induced resistance decrease would range between 1500 and 6500 Mohm, which value is much higher than the membrane resistances reported by Werblin(1978) and by Bader et al. (1978). For rat rods, which are much smaller than toad rods, Hagins and Yoshikami (1975) report values for the dark current between 20 and 70 pA. Related to the rather diverging dimensions of rat rods and toad rods the respective membrane resistances (ohm cm^2) would differ by more than one order of magnitude It may be relevant in this respect to point to the metabolic consequences (c.f. Hagins et al , 1970) of the fact that the membrane potential of rod and cone cells in the dark and of rod cells also in the light is 30-60 mV displaced from the K^+ equilibrium potential From the foregoing it will have become clear that under most medium and light conditions used the major light-independent conductance(s) carries a current of K^+ ions. A resistance of 10 Mohm at slightly depolarized membrane potentials in rods and a K^+ equilibrium potential of -80 mV (Werblin, 1979, Fain et al., 1978) would infer a K^+ current of about 6 nA Assuming that the dimensions of the mudpuppy rod cell (used by Werblin, 1979) are 12 x 100 μm, the pumping capacity necessary to maintain the ionic gradients would consume 1.8 mM ATP/rod cell per s (a turnover of three K^+ ions per ATP hydrolyzed is assumed). This value seems unrealistically high.

In conclusion, the experimental data available on the ionic channels and ionic fluxes in rod cells contain a number of serious discrepancies. In addition, the experimental data are not in accordance with the simple model that strong lights cause the blocking of all available Na^+ channels in the rod outer segment.

SUMMARY

Photoreceptor cells convert an electromagnetic input signal into an electrochemical output signal. A photon is absorbed by a photopigment molecule, which is embedded in a phospholipid bilayer membrane. This event leads to a modified ion current through ionic channels, which are embedded in a phospholipid bilayer membrane as well. In rod cells, the photopigment molecules and the ionic channels are located in different membranes, which are thought to be separated by an aqueous compartment, the rod cytosol. In order to bridge the latter it was suggested that bleaching of a photopigment molecule, located in the disk membrane, results in the production of an intermediate substance (transmitter), which in the rod cytosol diffuses to and interacts with the ionic channels, located in the plasma membrane. This model has dominated the last decade and at the time this investigation was started experiments were almost exclusively designed to supply supporting evidence for the hypothesis that the transmitter molecules are Ca^{2+} ions. These Ca^{2+} ions were thought to be released from the disk interior upon illumination and would subsequently block the channels, through which the dark current of Na^{+} ions is considered to pass.

In contrast to the simplicity of this hypothesis, it proved difficult to obtain reproducible and meaningful data on the Ca^{2+} content of rod outer segment disks and its possible dependence on light. Since hardly anything was known on the metabolic machinery, underlying the proposed role of Ca^{2+} ions, this investigation was started by analyzing in depth the problem in working with isolated rod outer segment preparations. As criteria for their structural integrity the state of the plasma membrane and of the stacking pile of disks have been used (chapter II), while functional integrity has been considered to be reflected by their capability to store Ca^{2+} ions and to transport Ca^{2+} and Na^{+} ions (chapters I and IV).

Along these lines procedures were devised to stabilize cattle rod outer segments, in order to allow purification of outer segments with an intact plasma membrane as well as to allow ample experimental manipulations without harming the integrity of the plasma membrane and of the stacked pile of disks (chapter II). From the literature (see chapter II) and from chapter I

one may conclude that these points are essential. In order to appreciate the improvement achieved with this preparation as compared to the highly deficient state of conventional rod outer segment preparations, it may be noted that conditions, which affect the stability and structural integrity of isolated rod outer segments, always cause a parallel deterioration of cation transport and storage systems of rod outer segments (chapter III and IV).

Using this new preparation it was found that Ca^{2+} ions in isolated (cattle) rod outer segments are predominantly stored by binding (> 99%) at intradiskal (> 60-70%) binding sites of high capacity and relatively high affinity (chapter IV). The intradiskal membrane phase behaves as a cation exchange membrane at which Ca^{2+} ions (as well as other divalent cations) and protons can be reversibly exchanged. In chapter III it is shown that bleaching of rhodopsin molecules in the disk membrane results in abolition of part of the Ca^{2+}-binding capacity, but that Ca^{2+} ions are not released across the disk membrane unless a Ca^{2+}-carrier (A23187) is added. It is concluded that these observations are not easily reconciled with the proposed role of Ca^{2+} ions as intracellular transmitter.

In chapters IV and V an endogenous cation exchange system was discovered and characterized which makes the intracellular Ca^{2+} ions rapidly (seconds) accessible to the extracellular space by a pre ominantly electroneutral exchange transport (chapters IV and V). Unlike cation transport, induced by exogenous ionophores (Gramicidin, A23187), the endogenous cation transport system sharply discriminates between the physiologically occuring cations in a complex manner, which shows a number of correlates with the system responsible for the electrical properties of the rod cell in the retina (chapter V and section 3.3). The presence of ionic channels with distinctive selectivity ranges, especially for cations, is generally considered implicit to the functioning of excitable membranes. In rod cells (sections 2.1, 2.2, and 2.3) and in isolated rod outer segments (chapter V) a system is present, which transports Na^+ and Ca^{2+} ions and which absolutely discriminates between Li^+ and Na^+ ions. This marks a clear distinction with the well-known Na^+ channels in axon membranes.

At the present state of available data it is difficult to attribute to the cation transport system in rod outer segments a channel (pore)-type or carrier-type of molecular mechanism, since it combines properties of both.

Thus, the junctional transport route between disks and plasma (chapter V) and the low Q_{10} (1 08) of transport, about equal to aqueous diffusion, suggest a channel mechanism. On the other hand, the phenomenon of exchange diffusion, competitive inhibition and titratable ionic sites are generally associated with carrier mechanisms. In any case, the control of the transport parameters (rate and affinity constants) in a way, which suggests that they depend on the membrane potential, and the cooperativity of individual transport systems (chapter V) furt'er illustrate the versatility of the cation transport system in rod outer segments.

Growing understanding of the biochemistry of ions in rod outer segments should be critically compared with other approaches. This is attempted in sections 1 to 4 of this thesis. Since the present concepts and hypotheses on the role of ions in visual excitations are largely derived from electrophysiology, special attention is given to studies from this discipline.

In the sections 2 and 4 the electrophysiology of rod and cone cells is discussed and the requirements, which a hypothetical transmitter should meet, are analyzed. The kinetic analysis of photoresponses has suggested that the transmitter concentration increases linearly with the light intensity and gives rise to local effects on local illumination at all light intensities. Quantal responses are uniform in amplitude and kinetics, which infers a spatial and temporal homogeneous distribution of transmitter molecules irrespective of the locus of the bleachedpnotopigment molecule. Adaptation to background lights is most likely correlated to the steady state transmitter concentration. Conditions, which are suggested to cause changes in the steady state concentration of the two candidates for the intracellular transmitter (i.c. Ca^{2+} ions and cGMP), affect the peak voltage of the photoresponses, but have only minor effects on the time scale and sensitivity of photoresponses. Hence, the conclusion seems obvious that both Ca^{2+} ions and cGMP are most likely not the intracellular transmitter produced by light Sections 2 3 and 2.4 discuss a modified proposal for the case of Ca^{2+} ions.This might obviate the objections mentioned above and the fundamental objections against the presence of any diffusable transmitter in the cytosol, formulated in section 3. The properties of the Na^{+}- and Ca^{2+}-sensitive transport system of rod outer segments, notably the junctional transport route between individual disks and the plasma membrane and t'e cooperativity between individual junctions, inspired us to outline a sketch for a new scheme, which essentially proposes a transduction process within the disk membranes (section 3.3).

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Chapter I Schnetkamp, P.P.M., Daemen, F.J.M. and Bonting, S.L. (1977)

Biochemical aspects of the visual process XXXVI. Calcium accumulation in cattle rod outer segments: evidence for a calcium-sodium exchange carrier in the rod sac membrane.

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Light-induced calcium release in isolated intact cattle rod outer segments upon photoexcitation of rhodopsin.

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Ion selectivity of the cation transport system of isolated intact cattle rod outer segments: evidence for a direct communication between the rod plasma membrane and the rod disk membranes.

Biochim.Biophys.Acta, in press

CURRICULUM VITAE

Paul P.M. Schnetkamp werd op 29 juni 1949 geboren te 's Hertogenbosch. In 1967 werd het eindexamen gymnasium bêta behaald (St. Janslyceum te 's Hertogenbosch) en werd begonnen met de scheikunde studie aan de Universiteit te Nijmegen. In 1970 werd het kandidaatsexamen S_2 behaald en in juli 1974 volgde het doctoraalexamen scheikunde (hoofdvak: biochemie; bijvakken: theoretische chemie en chemische cytologie). Van 1 juli 1974 tot 1 april 1978 is hij werkzaam geweest aan de afdeling biochemie van de Medische Faculteit van de Universteit te Nijmegen, waar het hier beschreven promotie onderzoek is verricht onder leiding van Prof.Dr. F.J.M.Daemen en Prof.Dr. S.L.Bonting. Sinds mei 1978 is hij werkzaam in de werkgroep van Prof.Dr. W.Junge, tot augustus 1979 aan de Technische Universitat Berlin en sindsdien aan de Universitat Osnabruck.

CALCIUM IONS AND VISUAL EXCITATION

P.P.M. SCHNETKAMP

STELLINGEN

I

De kinetiek en dynamiek van de fotosignalen van vertebraat staafjes en kegeltjes cellen lijken moeilijk in overeenstemming te brengen met de algemeen aanvaarde hypothese, dat een diffunderende transmitter in het cytoplasma van deze cellen het transduktie proces verklaart.
Dit proefschrift, sektie 3.

II

De experimenten, die als ondersteuning worden aangevoerd voor de hypothese, waarin calcium ionen de transmitters zijn in staafjes cellen, lijken uit te sluiten, dat deze calcium ionen door licht worden vrijgemaakt uit de staafjes buitensegment disks.
Dit proefschrift, sektie 2.3.

III

Er is geen overtuigend experimenteel bewijs voor het algemeen gehanteerde model, waarin absorptie van licht leidt tot de sluiting van alle natrium kanalen in staafjes buitensegmenten.
Dit proefschrift, sektie 4.3.

IV

Bij de diskussie over de primaire fotochemische stap in de bleking van rhodopsine lijkt het een zinvolle vraag in hoeverre voor individuele rhodopsine molekulen een temperatuur van $4^{o}K$ op een tijdschaal van picosekonden gedefinieerd is, indien 22 Kcal/mol van een geabsorbeerd foton als warmte vrijkomt.
Peters, K., Applebury, M.L. en Rentzepis, P.M. (1977) Proc.Nat.Acad. Sci. 74,3119-3123.
Honig, B., Ebrey, T., Callender, R.H., Dinur, U. en Ottolenghi, M. (1979) Proc.Nat.Acad.Sci. 76,2503-2507.
Cooper, A. (1979) Nature 282,531-533.

V

Het experimentele systeem, op basis waarvan Hong en Mauzerall de "chemische kapaciteit" postuleren, bevat belangrijke onduidelijkheden.
Hong, F.T. en Mauzerall, D. (1974) Proc.Nat.Acad.Sci. USA 71, 1564-1568.
Hong, F.T. (1976) Photochem. Photobiol. 24, 155-189.

VI

De konklusie van Flaming en Brown, dat de verandering van de lichtgevoeligheid van staafjes cellen door verandering van de extracellulaire calcium koncentratie wijst op een adapterende rol van calcium ionen, en niet op een rol als transmitters, is niet in overeenstemming met hun experimentele gegevens.
Flaming, D.G. en Brown, K.T. (1979) Nature 278, 852-853.

VII

De bewering van Deutsch en Rafalowska, dat de verdeling van trifenylmethylfosfonium ionen in synaptosomen bepaald wordt door de transmembraan potentiaal en dat de oppervlakte en interface potentialen geen rol spelen, is onvoldoende experimenteel onderbouwd.
Deutsch, C. en Rafalowska, U. (1979) FEBS Lett. 108, 274-278.

VIII

De methode, waarmee Tiemann et al. de licht-geïnduceerde protonen opname door thylakoiden bepalen, leidt tot foutieve resultaten.
Tiemann, R., Renger, G., Graber, P. en Witt, H.T. (1979) Biochim. Biophys. Acta 546, 498-519.

IX

De manier, waarop Lamb en Simon uit de door ruisanalyse verkregen diskrete veranderingen van de membraan potentiaal de geleidbaarheid van individuele ionenkanalen berekenen, kan slechts verklaard worden als een akuut en gelukkig slechts plaatselijk geval van beroepsblindheid.
Lamb, T.D. en Simon, E.J. (1977) J. Physiol. 272, 435-468.

X

Bij de interpretatie van de effekten van de intracellulaire injektie van cyklisch GMP in staafjes cellen hebben Miller en Nicol zich duidelijk laten leiden door het motto, dat de wens de vader van de gedachte is.
Miller, W.H. en Nicol, G.D. (1979) Nature 280, 64-66.

XI

Het strekt tot aanbeveling de betrouwbaarheid van experimentele gegevens aan te geven in de volgende aflopende reeks S.D. > S.E. > S.F.

Nijmegen, 29 februari 1980 Paul Schnetkamp